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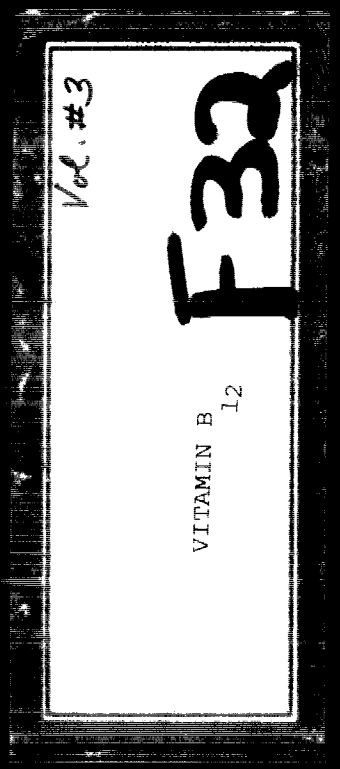

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ESTIMATION OF THE TOTAL BODY VITAMIN B_{12} IN THE LIVE SUBJECT

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SUMMARY

1. Values for total body vitamin B_{12} were calculated for eighteen patients by giving a tracer dose of radioactive cyanocobalamin and measuring the radioactivity and microbiological activity in liver biopsies obtained at laparotomy.

2. The values for total body vitamin B_{12} ranged from 960 to 5984 μg with a mean of 2528 μg. Five values were greater and thirteen less than the mean.

3. Significant correlations were found between the serum vitamin B_{12} and total body vitamin B_{12}, the serum vitamin B_{12} and the hepatic vitamin B_{12} and the hepatic B_{12} and the total body vitamin B_{12}.

Work published to date on the vitamin B_{12} content of the body relies mainly on calculations based on microbiological analysis of tissues obtained post mortem from patients with a wide variety of diseases. Mean values of 2689 μg, 3900 μg and 5000 μg with ranges of 1634 to 3475 μg, 790 to 11,100 μg and 3480 to 10,950 μg have been suggested by Kurlov (1961, 1962), Gräsbeck, Nyberg & Reizenstein (1958) and Heinrich (1964) respectively, and Adams (1962) reported a mean of 2221 μg with a range of 953–4304 μg from a combined isotope dilution and microbiological assay procedure.

The only report on the vitamin B_{12} content of the body in the living subject is one by Reizenstein, Ek & Matthews (1966) in which an average value of 3030 μg was obtained by kinetic analysis of values for whole body retention, faecal excretion and plasma clearance after parenteral radioactive vitamin B_{12}. We are not aware of any other estimates of total body vitamin B_{12} in the living subject and so we report here our experience with a procedure by which approximate values were obtained by relatively simple methods in this situation. Although limited in scope and application we believe it has a place in studies of vitamin B_{12} metabolism in man.

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MATERIALS AND METHODS

The basic principle is that of isotope dilution which assumes that, at a finite time after administration, a tracer dose of radioactive material is distributed throughout the body in proportion to the endogenous non-radioactive material. Previous work suggests that equilibration of an intravenously administered dose of 100 ng radioactive cyanocobalamin with body stores of vitamin B_{12} occurs 5 to 10 days after injection and that the loss of radioactive material from the body between the time of injection and equilibration is of the order of 5% (Adams & Boddy, 1968). Thus the total amount of vitamin B_{12} in the body can be calculated by measurement of radioactivity and vitamin B_{12} content of a suitable tissue obtained after equilibration and by allowing for the loss of radioactive material from the body.

On this basis patients referred for elective surgery were appraised of the nature of the study and, after a sample of blood had been obtained for serum vitamin B_{12} estimation, were given 50 ng 5·0 μCi [^{57}Co]cyanocobalamin in 5·0 ml water intravenously. At operation, which was performed 6–29 days later, a wedge of liver was obtained, usually from the antero-inferior aspect of the left lobe, and haemostasis secured by sutures before the major procedure. The tissue was weighed in a Stanton analytical balance and stored at $-20°$ if necessary before homogenization with water in a Potter Elvehjm or Silverson microhomogenizer to a volume of 20 ml. The activity was measured in a well-type scintillation counter IDL type 663 with a thallium activated sodium iodide crystal, 5·5 cm. diameter and 6·9 cm. deep, shielded by 10·0 cm lead and connected to an IDL 1700 automatic scaler using three standards each containing 1·0% of the dose in 20 ml water. At least 10,000 counts were obtained from every sample. The homogenate was then further homogenized with water in a Waring Blender to suitable dilutions for microbiological assay by the method of Hutner, Bach & Ross (1956) using Euglena gracilis Z strain as the test organism, and commercially available medium (Difco Laboratories Inc.). The homogenate and serum sample from each patient were assayed together on at least three occasions.

[^{57}Co]cyanocobalamin was obtained from the Radiochemical Centre, Amersham, two batches being used in the study. The material was dissolved in distilled water and dispensed in 5 ml aliquots in dark glass ampoules after sterilization by Millipore filtration. The ampoules were stored at $+4°$. Before use of a batch an ampoule was taken at random and the purity of the solution tested by column chromatography using carboxy-methyl-cellulose and diethyl-amino-ethyl-cellulose. The final ampoule in each batch was tested similarly. The rationale of these procedures, described in detail by Kennedy & Adams (1965) & Kennedy (1967) is that cyanocobalamin, being neutral, is not retained by either material whereas hydroxocobalamin, which results from photolysis of cyanocobalamin (Smith, 1965), is retained by the cation exchanger carboxy-methyl-cellulose and the 'red acids', which result from radiochemical decomposition of cyanocobalamin (Smith, 1965), are retained by the anion exchanger diethyl-amino-ethyl-cellulose.

RESULTS

Eighteen patients were studied, the relevant information on each being given with the essential results in Table 1. There was no morbidity in the series. There was no evidence, by the methods used, that the [^{57}Co]cyanocobalamin solutions tested had deteriorated during storage.

TABLE 1. Showing details of patients and essential results

Case	Age	Sex	Serum B_{12} (pg/ml)	Disease and operation	Time interval between injection and biopsy (days)	Liver biopsy Mass (g)	Liver biopsy activity (% dose)	Assayable B_{12} (μg)	Calculated 'Total Body B_{12}' (μg)
1	20	M	463	Duodenal ulcer—partial gastrectomy	29	3·7210	0·2389	6·1305	2438
2	47	F	—	Gastric ulcer—partial gastrectomy	25	5·4507	0·3832	6·7689	1678
3	59	M	433	Gallstones—cholecystectomy	18	4·1903	0·1979	3·8255	1836
4	67	M	191	Gallstones—cholecystectomy	22	5·0027	0·2184	3·1650	1377
5	74	F	511	Gallstones—cholecystectomy	6	4·4167	0·2867	7·7263	2560
6	64	M	490	Gallstones—cholecystectomy	11	2·0411	0·1017	1·0526	983
7	57	M	444	Duodenal ulcer—gastroenterostomy and vagotomy	15	2·5576	0·1247	2·0680	1575
8	53	M	353	Gallstones—cholecystectomy	14	2·3975	0·0644	1·6156	2383
9	41	F	403	Duodenal ulcer—pyloroplasty and vagotomy	14	3·0041	0·1245	2·5530	1948
10	33	M	540	Duodenal ulcer—pyloroplasty and vagotomy	10	4·1765	0·1878	3·9050	1975
11	46	M	535	Duodenal ulcer—gastroenterostomy and vagotomy	14	2·2592	0·0634	2·1439	3212
12	52	F	617	Cholecystitis—cholecystectomy	15	3·9550	0·1812	4·5601	2391
13	26	M	670	Duodenal ulcer—gastroenterostomy and vagotomy	18	2·7906	0·0715	4·5040	5984
14	54	F	585	Gallstones—cholecystectomy	22	2·4200	0·0874	1·8972	2062
15	43	F	286	Crohn's disease—resection of intestinal stricture	20	2·2201	0·0896	0·9058	960
16	52	M	607	Duodenal ulcer—pyloroplasty and vagotomy	29	2·2922	0·0535	3·3145	5886
17	56	M	333	Gastric ulcer—partial gastrectomy	28	2·8689	0·1029	3·3508	3093
18	37	M	350	Cholecystitis—cholecystectomy	24	2·1527	0·0541	1·8018	3164

The amount of radioactivity in the liver biopsies ranged from 0·0535 to 0·3832% of the dose and the microbiological activity expressed as cyanocobalamin ranged from 0·9058 to 7·7263 μg per biopsy and from 0·41 to 1·75 μg/g tissue, mean 1·06 μg/g. The total body vitamin B_{12} values were calculated from the values for radioactivity and vitamin B_{12} in the biopsies assuming retention of 95% of the dose and ranged from 960 to 5984 μg with a mean of 2528 μg: five values were greater than the mean and thirteen less than the mean. Serum vitamin B_{12} values were estimated in seventeen subjects (collection of the sample from one patient having been omitted by accident) and ranged from 191 to 670 pg/ml, mean 459 pg/ml.

Significant correlations were found between the values for serum vitamin B_{12} in pg/ml (x) and total body vitamin B_{12} in μg (y) in seventeen patients the regression equation being:

$$y = 6{\cdot}0634\,x - 207{\cdot}8946 \quad (r\ 0{\cdot}54;\ P < 0{\cdot}05)$$

and between the values for serum vitamin B_{12} in pg/ml (x) and hepatic vitamin B_{12} in μg/g of tissue (y) in seventeen patients the regression equation being:

$$y = 0{\cdot}001674\,x + 0{\cdot}2357 \quad (r\ 0{\cdot}53;\ P < 0{\cdot}05)$$

and between the hepatic vitamin B_{12} in μg/g tissue (x) and the total body vitamin B_{12} in μg (y) in eighteen patients the regression equation being:

$$y = 2250{\cdot}4866\,x - 237{\cdot}0602 \quad (r\ 0{\cdot}63;\ P < 0{\cdot}01).$$

DISCUSSION

The validity of the results depends on three premises. First, that the ^{57}Co cyanocobalamin had equilibrated with the non-radioactive vitamin B_{12} in the body when the liver biopsy was obtained. Second, that the microbiological assay procedure using cyanocobalamin standards measured the liver vitamin B_{12}. Finally that the use of an arbitrary value, in this case 95%, for dose of tracer retained in the body at the time of the liver biopsy is acceptable for calculation of total body vitamin B_{12} values.

Probably the most contentious of these premises is the first. There is controversy as to whether orally or parenterally administered radioactive vitamin B_{12} does, or does not, equilibrate with the non-radioactive stores in the body. Reizenstein, Matthews & Ek (1964), Reizenstein, Matthews & Ek (1966) and Schiffer, Cohn, Price & Crunkite, (1968) do not consider that equilibration occurs within a finite time and the first two groups of workers link this opinion with the concept of at least three pools in the body. Heinrich (1964) while also favouring a multipool system accepts that equilibration occurs but only after 240–300 days after administration of radioactive vitamin B_{12}. Others have concluded from studies in animals that equilibration occurs more rapidly (Cooperman, Luhby, Teller & Marley, 1960; Grasbeck, Ignatius, Jarnefelt, Linden, Mali & Nyberg, 1961; Newman, O'Brien, Spray & Witts, 1962) and similar conclusions have been reached from studies in man (Bozian, Ferguson, Heyssell, Meneely & Darby, 1963; Heyssel, Bozian, Darby & Bell, 1966; Boddy & Adams, 1968; Adams & Boddy, 1968). With the exception of the time scale suggested by Heinrich (1964) the differences of opinion about the time taken for equilibration are less contentious being related, in part at least, to the mass of the dose given and possibly also to the species studies. In humans given 5000 μg doses parenterally the loss of whole body radioactivity did not occur at a steady rate, which was taken as evidence of equilibration, for several weeks (Boddy & Adams, 1968), whereas with doses of 100 ng parenterally the rate of loss was steady after 5–10 days (Adams & Boddy 1968).

We feel that the measurement of liver vitamin B_{12}, which is mainly coenzyme B_{12} (Toohey &

Barker, 1961; Ståhlberg, Radner & Nordén, 1967), by microbiological assay using cyanocobalamin standards is acceptable, our opinions being based on the results of recovery studies with coenzyme B_{12} added to liver homogenates *in vitro*. The values for vitamin B_{12} are greater than those found by Pitney, Beard & Van Loon (1955) and Jhala & Gadgil (1960) but are comparable to those reported by Blum & Heinrich (1957), Ross & Mollin (1957), Pitney & Onesti (1961), Adams (1962), Joske (1963), Anderson (1965) and Ståhlberg *et al.* (1967) also using *Euglena gracilis* as the test organism.

The use of an arbitrary value for the proportion of dose retained in the body at the time of liver biopsy was based on data reported by Adams & Boddy (1968). In this study the loss of whole body radioactivity by normal subjects given 100 ng [^{57}Co]cyanocobalamin intravenously was initially rapid but after 5–10 days, by which time about 5% had been lost, settled to a rate of 0·1–0·2% per day. We felt, therefore, that a suitable overall allowance for loss in the time between administration of the smaller dose used in this study and liver biopsy would be 5%. The ideal procedure, of course, would be to use a whole body monitor to obtain a 100% value after administration of the tracer dose and to repeat the measurement on the day of operation to obtain a value for retained dose. Consideration of the values involved, however, makes it doubtful if the results would be materially affected by such a procedure even given a monitor with the sensitivity and performance required in such a situation. In view of the doubts, however small, which must always attend the use of arbitrary values we regard it as prudent to regard the estimates of total body vitamin B_{12} obtained by this method as approximate values.

Of the correlations between serum, hepatic and total body vitamin B_{12} which we report, two are novel and one, that between serum and hepatic vitamin B_{12}, complements the finding by Anderson (1965) of a correlation between these measurements in a large series of vitamin B_{12} deficient patients and the circumstantial evidence for a relationship adduced by Chanarin (1969) from selected data on vitamin B_{12} deficient and vitamin B_{12} replete subjects studied by Joske (1963), Anderson (1965) and Ståhlberg *et al.* (1967). In his own results Joske (1963) did not find a correlation probably because, as he states, the results were obtained mainly from patients with parenchymal liver disease which may upset the balance between the serum and hepatic vitamin B_{12}. We think it unwise at present to draw any conclusions from the correlations other than the general consideration that the serum vitamin B_{12} per unit of volume, the hepatic vitamin B_{12} per unit of mass and the total body vitamin B_{12} would appear to be related to each other.

Whether the values for total body vitamin B_{12} we report can be regarded as representative of normality is conjectural. Certainly we have doubts about regarding a patient with Crohn's disease as normal from the point of view of vitamin B_{12} metabolism and the fact that patients with duodenal ulcers have a higher than normal output of intrinsic factor in response to histamine (Rødbro, Christiansen & Schwartz, 1965) might raise doubts about this group. In this connection it may be relevant to note that the mean total body vitamin B_{12} for the seven patients with duodenal ulcers was 3288 μg and for the six patients with gall stones was 1867 μg. These values are significantly different when analysed by the Mann-Whitney 2 tail test (P = 0·014) but the mean value for the ulcer patients is heavily loaded by inclusion of two very high results and when these are excluded the significance disappears (P = 0·53). Within these limitations and the obvious problems associated with the study of normal subjects we feel that the results at least provide material of relevance and interest in studies of vitamin B_{12} metabolism in man.

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Interrelation of Serum Vitamin B_{12}, Total Body Vitamin B_{12}, Peripheral Blood Morphology and the Nature of Erythropoiesis

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SUMMARY. The interrelation of serum vitamin B_{12} concentration, body vitamin B_{12}, peripheral blood morphology and the nature of erythropoiesis was studied in patients with pernicious anaemia. On presentation the patients were treated with radioactive vitamin B_{12} and the retained radioactivity, measured by whole body monitoring, was taken as a measure of the body vitamin B_{12}.

Post-treatment results suggest that erythropoiesis became megaloblastic when the serum vitamin B_{12} was between 70 and 154 pg/ml and usually when the body vitamin B_{12} was between 100 and 660 μg and that the serum vitamin B_{12} was of more immediate importance in the nature of erythropoiesis than the body vitamin B_{12}.

A normal peripheral blood film was no indication of the nature of erythropoiesis but an abnormal film carried a high probability of megaloblastic erythropoiesis. Abnormalities in blood films probably developed after abnormalities in marrow.

The correlation found between serum vitamin B_{12} and body vitamin B_{12} differed from that found in controls and the mean quotient of body vitamin B_{12} and serum vitamin B_{12} concentration was less than in controls showing that, in the patients studied, there was a disproportionately greater amount of vitamin B_{12} in serum compared to controls.

The balance of serum vitamin B_{12} and body vitamin B_{12} may involve at least two mechanisms and folate may have a role in both. One mechanism relates to the release of vitamin B_{12} from stores to plasma and the other to the clearance of vitamin B_{12} from plasma.

The two most important factors which appear to determine whether erythropoiesis is normoblastic or megaloblastic are the vitamin B_{12} and the folate status of the individual. Insofar as vitamin B_{12} status is concerned, there is evidence relating the nature of erythropoiesis to the serum vitamin B_{12} concentration and evidence of a relationship between the serum vitamin B_{12} concentration and the body stores of vitamin B_{12} but, beyond this, little is known of the interrelationship of these factors.

The object of the work reported in this paper was to study this interrelationship and for this purpose the body vitamin B_{12} was determined by measurements of whole body radioactivity in patients who, on presentation with vitamin B_{12} deficiency, had been treated with

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radioactive vitamin B_{12}, the retained fractions of which were taken to constitute their body stores for practical purposes.

MATERIALS AND METHODS

Patients. The investigations were made on 22 patients with pernicious anaemia. Because the study involved interruption of treatment the nature and objects were carefully explained and informed consent obtained. Most patients had presented with symptoms of anaemia but some came under observation with symptoms suggesting vitamin B_{12} deficiency such as glossitis or paraesthesiae and one (Case 19) had long tract signs on presentation. Before treatment all patients had a low serum vitamin B_{12} concentration and megaloblastic erythropoiesis and were later found to have histamine or pentagastrin fast achlorhydria and malabsorption of radioactive cyanocobalamin correctable by intrinsic factor.

Clinical procedures. Twelve patients were treated with a single intravenous dose of radioactive cobalamin, four with five or six daily intravenous doses and six with two intravenous doses at intervals of 1 or 6 weeks. Only one patient (Case 20), who was also given intramuscular iron, had any other treatment. After treatment patients were seen at monthly intervals for clinical examination, blood sampling and whole body monitoring. The duration of individual study varied from 84 to 456 days and was determined by peripheral blood values, serum vitamin B_{12} concentration, the amount of the dose retained and radioactive decay.

Radioisotope methods. Radioactive cyanocobalamin and hydroxocobalamin were obtained from the Radiochemical Centre, Amersham, and radioactive 5′ deoxyadenosyl cobalamin (coenzyme B_{12}) was prepared from radioactive hydroxocobalamin by Dr L. Mervyn of Glaxo Ltd by the method of Johnson *et al* (1963). The cobalamins were labelled with ^{57}Co except in the case of six subjects who were given ^{58}Co cyanocobalamin followed by ^{57}Co hydroxocobalamin. Particular care was taken to avoid photolysis of coenzyme B_{12} preparations.

Whole body monitoring was effected using the Merlin whole body monitors (Boddy, 1967a, b). Before treatment the radioactivity due to natural potassium 40 and caesium 137 was measured and a further measurement after the initial cobalamin dose gave a value corresponding to 100% retention in those given one dose and an incremental value in those given multiple doses. All counting rates were corrected for background, natural body radioactivity and radioactive decay.

Haematological methods. Haemoglobin and packed red cell volume estimations and blood films were made at each attendance and marrow biopsy was performed at the last attendance. All pre- and post-treatment marrow smears were collected and submitted to one observer (A.S.D.) who had not seen them previously and who was unaware of any data, clinical or otherwise. Unknown to the observer, preparations of relevance accounted for only half the material submitted, the remainder being drawn for other patients with untreated or treated megaloblastosis not included in the study. Slides were identifiable by code number only. Erythropoiesis was graded as normoblastic (N) or megaloblastic (M), the degrees ranging from minimal (M) to florid (M+ + + + +). A similar procedure was followed with post-treatment blood films which were submitted using a different code several months

later. The films were reported as normal (0) or abnormal, the degree ranging from minimal (+) to marked (+ + +).

Measurement of total body vitamin B_{12}. In nine patients in whom relevant data were available the pretreatment body vitamin B_{12} was measured by the method of Boddy & Adams (1972). After treatment the body vitamin B_{12} was calculated in microgrammes from the fraction of the administered radioactive material retained in the body, no allowance being made for stores before treatment.

Microbiological methods. Two aliquots of serum were collected at each attendance and stored at −20°C. One was assayed soon after collection and the other stored until the end of the individual study when all samples from the patient were assayed en bloc on three separate occasions by the method of Hutner *et al* (1956) using *Euglena gracilis* Z strain as the test organism. Serum folate levels were measured by microbiological assay using *Lactobacillus casei* as described by Waters & Mollin (1961). The normal range in this hospital is from 3.2 to 17.6 ng/ml.

RESULTS

Relevant pretreatment data, treatment schedules and post-treatment data are shown in Table I. Serum folate levels were normal or high before treatment and normal at the end of the study (Table I). Iron status, assessed by amounts of iron in final marrow sections, was considered to be subnormal in Cases 2, 6, 8–13, 15–17 and 21 and normal in the others.

Clinical

Symptoms were alleviated by treatment in all cases and did not recur in any patient during the period of study. The long tract signs in Case 19 remained unchanged but calf tenderness disappeared and did not recur.

Haematological

All anaemia patients had a haematological response to treatment with rise in peripheral blood values to normal and these were maintained during the period by all except Case 20 whose haemoglobin fell from 12.0 g/100 ml to 9.5 g/100 ml in the last 8 weeks.

Of the 26 post-treatment marrow smears erythropoiesis was judged to be megaloblastic in 14 and normoblastic in 12 instances. In the normoblastic group the serum vitamin B_{12} levels ranged from 70 to 610 pg/ml and the body vitamin B_{12} values from 100 to 1200 μg; in the megaloblastic group the serum vitamin B_{12} levels ranged from 70 to 154 pg/ml and the body vitamin B_{12} values from 100 to 1460 μg, one value (Case 2) being greater than 660 μg.

Post-treatment blood films were considered to be normal in 18 instances and abnormal in eight. In the normal film group the corresponding marrow smears showed normoblastic erythropoiesis in 10 and megaloblastic erythropoiesis in eight instances. In the abnormal film group erythropoiesis was normoblastic in two and megaloblastic in six instances.

Statistical

A linear correlation was found between the values for serum vitamin B_{12} (x) and body

TABLE I. Relevent details before and after treatment with radioactive cyanocobalamin (CN B_{12}), hydroxocobalamin (OH B_{12}) or deoxyadenosylcobalamin (coenzyme B_{12}, CoE B_{12}). Under the heading of 'marrow', erythropoiesis is classified as N (normoblastic) or M (megaloblastic) and under the heading of 'film', peripheral blood morphology is classified as o (normal) or + (abnormal). In the six cases given two cobalamins at intervals of 1 or 6 weeks the identification of the fractions retained as CN B_{12} or OH B_{12} is for convenience only and does not imply that the administered cobalimins were not converted to other forms.

Case	Age	Sex	Post-treatment					Treatment	Pretreatment						Dose retained %	Dose retained µg	$\left[\frac{\text{Body } B_1}{\text{Serum } B_{12}}\right]_k$
			Hb (g/100 ml)	Marrow	Serum B_{12} (pg/ml)	Body B_{12} (µg)	Serum folate (ng/ml)		Time (days)	Hb (g/100 ml)	Blood film	Marrow	Serum B_{12} (pg/ml)	Serum folate (ng/ml)			
1	50	F	9.7	M++	<25	65	2.4	6 × 5000 OH B_{12}	378	12.4	o	N	344	2.8	3.9	1170	3.4
2	62	M	8.5	M++++	<25	235	16.0	5 × 5000 OH B_{12}	434	14.1	+	M+	134	4.6	5.9	1475	11.0
3	87	F	11.2	M++	40	—	10.8	5 × 5000 OH B_{12}	222	13.5	+	N	557	9.2	4.8	1200	2.2
4	67	F	12.8	M++	80	—	2.8	5 × 5000 OH B_{12}	308	13.5	o	N	361	4.4	4.7	1175	3.3
5	61	F	12.0	M++	<25	—	14.6	5000 CN B_{12} / 5000 OH B_{12} 1/52	217	15.0	o	M+	154	6.8	4.8 CH 240 / 7.3 OH 365	605	3.9
6	69	F	5.6	M+++	<25	—	12.0	5000 CN B_{12} / 5000 CN B_{12} 1/52	217	13.9	o	N	118	11.6	5.4 CN 270 / 12.9 OH 645	915	7.8
7	63	F	13.1	M+	39	—	13.2	5000 CN B_{12} / 5000 OH B_{12} 1/52	255	14.2	o	M	79	6.6	2.7 CN 135 / 10.1 OH 505	640	8.1
8	44	F	6.8	M++	25	—	4.0	5000 CN B_{12} / 5000 OH B_{12} 6/52	265	14.2	o	N	129	4.8	4.1 CN 205 / 4.8 OH 240	445	3.5
9	58	F	13.3	M+++	55	—	9.6	5000 CN B_{12} / 5000 OH B_{12} 6/52	282	14.2	o	N	117	—	3.4 CN 170 / 8.1 OH 405	475	4.9
10	45	M	10.2	M+++++	44	—	5.3	5000 CN B_{12} / 5000 OH B_{12} 6/52	308	14.1	o	N	218	—	5.2 CN 260 / 11.2 OH 560	820	3.8
11	67	F	10.8	M+	37	53	20.0	5000 CN B_{12}	287	13.3	o	M+	107	20.4	3.6	180	1.7
12	73	M	5.1	M+++	28	45	4.1	5000 CN B_{12}	344	13.6	o	M+	97	6.0	3.4	170	1.8
13	41	F	9.7	M+	91	—	3.5	5000 CN B_{12}	168	13.0	o	M+	85	4.0	5.2	260	3.1
14	65	F	6.3	M++++	<50	141	8.0	5000 CoE B_{12}	312	12.6	o	N	93	7.4	7.9	395	4.2
									396	14.1	o	N	135		7.1	355	2.6
15	54	M	6.0	M++++	<25	—	7.6	5000 CoE B_{12}	202	14.3	++	M+	114	5.0	13.2	660	5.8
									419	14.5	o	M+	110		9.6	480	4.4
16	60	F	6.6	M+	25	84	8.0	5000 CoE B_{12}	230	14.2	o	M+	129	8.0	10.9	545	4.2
									314	13.0	o	N	152		8.8	440	2.9
									456	14.0	+	M+	137		7.5	375	2.7
17	58	M	7.0	M+++++	50	41	4.8	2000 CN B_{12}	326	17.4	o	N	247	12.4	9.2	184	0.7
18	79	F	6.0	M+++++	38	43	—	2000 CN B_{12}	303	14.1	+	M+++	119	5.8	7.7	154	1.3
19	49	M	13.0	M++	<25	44	24.0	1500 CoE B_{12}	231	14.6	o	M+	87	16.4	9.0	135	1.6
20	61	F	6.2	M+++++	40	—	8.0	1000 CN B_{12}	166	9.5	+++	M++++	73	4.0	8.1	81	1.1
21	61	M	13.3	M+++	40	—	8.0	1000 CN B_{12}	127	15.2	+	N	69	7.4	10.6	106	1.5
22	66	M	9.6	M++	49	—	4.0	1000 CN B_{12}	84	12.9	+	M++	69	8.8	17.1	171	2.5

vitamin B_{12} (y) at the time of final marrow biopsy, the regression equation being $y = 2.2022x + 185.6665$ ($r = 0.63$, $P<0.01$).

DISCUSSION

Two limitations are apparent in the study. The first is that the values for post-treatment body vitamin B_{12} are necessarily underestimates as no allowance was made for the relatively small pretreatment stores. This had to be accepted in the absence of any other method but in practice is not of such moment as to materially affect the conclusions. The second relates to decisions on the normality or abnormality of blood films and marrow smears and does not require elaboration other than to re-emphasize the difficulties and that bias by knowledge of circumstances was eliminated.

Whether abnormalities in peripheral blood films precede or succeed abnormalities in erythropoiesis is not clear. Herbert (1962) reported that in one case abnormalities in blood films preceded the appearance of megaloblastosis but Eichner *et al* (1971) found the opposite in two cases. Our results suggest that when the film is normal there is an approximately even chance of erythropoiesis being normoblastic or megaloblastic but that when the film is abnormal there is a high probability (6 in 8) that erythropoiesis will be megaloblastic. More pronounced megaloblastic changes were found in the group with the abnormal film–abnormal marrow sequence than in the group with the normal film–abnormal marrow sequence, suggesting that the latter occurred at an earlier stage than the former and that changes in peripheral blood follow the development of megaloblastosis.

If the values from one subject (Case 2) with a high body vitamin B_{12} and low serum vitamin B_{12} are excluded the results imply that the transition from normoblastosis to megaloblastosis occurs when the serum vitamin B_{12} is in the region of 70–154 pg/ml and the body stores in the region of 190–660 μg. The values for both serum vitamin B_{12} and body vitamin B_{12} are in good accord with previous observations on the relationship of the serum vitamin B_{12} to the nature of erythropoiesis and with observations on the magnitude of the body stores in control and vitamin B_{12} deficient subjects. Mollin & Ross (1953) found megaloblastosis to occur with serum vitamin B_{12} levels of 50–120 pg/ml and normoblastosis with levels of 80–860 pg/ml; further study of patients with serum vitamin B_{12} levels between 80 and 200 pg/ml showed that they could be divided into two groups by the presence or absence of abnormalities in marrow smears and that the group with megaloblastic erythropoiesis had serum vitamin B_{12} levels of 80–130 pg/ml (Anderson, 1965). The values for body vitamin B_{12} ranged from 100 to 660 μg when one value of 1475 μg, considered later, is excluded. These fall between the 40–250 μg range found in patients with pernicious anaemia in haematological relapse (Boddy & Adams, 1972) and the lower limit of the 800–3500 μg range estimated for controls (Kurlov, 1961, 1962; Gräsbeck *et al*, 1958; Adams, 1962; Heinrich, 1964; Adams *et al*, 1970).

A correlation between the serum vitamin B_{12} and body vitamin B_{12} was found in controls (Adams *et al*, 1970), in patients with untreated and treated pernicious anaemia (Boddy & Adams, 1972) and again in this series. In the controls the regression equation was body vitamin $B_{12} = 6.06 \times$ serum vitamin $B_{12} - 207$ and in this series it was body vitamin $B_{12} = 2.2 \times$ serum vitamin $B_{12} + 185$. The difference in regression coefficients, which is

significant ($P<0.05$), can be related to and further explored by the quotient (k) of the body vitamin B_{12} and serum vitamin B_{12}. In untreated pernicious anaemia the mean value for the quotient was 2.97 ± 0.82 and in the present series it was insignificantly different at 3.23 ± 0.45, there being no difference between the means from cases with normoblastic erythropoiesis and those with megaloblastic erythropoiesis. Both these means are significantly less ($P<0.05$) than the 5.66 ± 0.59 in controls. The difference cannot be due to the entry of vitamin B_{12} from gut to plasma, which has been considered to be a factor in the maintenance of serum vitamin B_{12} levels (Chanarin, 1969) as this would give a higher k value in patients with pernicious anaemia when compared to controls. It therefore suggests a fundamental difference in the metabolism of vitamin B_{12} by patients with pernicious anaemia and by controls and implies that there is a disproportionately greater fraction of vitamin B_{12} in serum in pernicious anaemia, i.e. that the serum vitamin B_{12} level is maintained at the expense of stores. This was suggested by Mollin & Ross (1953) but was queried by Booth & Spray (1960) and Simnett & Spray (1962) after observations on gastrectomized rats. In patients with pernicious anaemia we found that when treatment was withheld the serum vitamin B_{12} level declined at an insignificantly different rate from the body vitamin B_{12} in most cases (Boddy & Adams, 1972). Although this could be interpreted as evidence against the concept of maintenance of serum level at the expense of stores it does not disagree with the present findings because changes in the quotient developing over the years between normality and vitamin B_{12} depletion would not have been obvious in a shorter time of that study.

An unusually high quotient (k) of body vitamin B_{12} and serum vitamin B_{12} could occur as a result of two different mechanisms. One would be unduly rapid clearance of vitamin B_{12} from plasma and the other failure to release vitamin B_{12} from stores to plasma. The first mechanism was considered to be responsible, in part at least, for the unusually high k value found in Case 2 (Boddy & Adams, 1972) who is also notable for reasons to be discussed later. Unduly rapid clearance of vitamin B_{12} from plasma might be a factor in the decline in serum vitamin B_{12} levels during normal pregnancies (Heinrich, 1954; Boger *et al*, 1957; Okuda *et al*, 1956; Zachau-Christiansen *et al*, 1962; Ball & Giles, 1964), the rapid clearance being dictated by active transport of vitamin B_{12} from maternal to foetal plasma as suggested by the higher levels in the latter, both when the maternal level is normal (Killander & Vahlquist, 1954; Boger *et al*, 1957; Okuda *et al*, 1956) and low (Adams, 1958).

There is no evidence to support the existence of the second mechanism—failure or depression of release of vitamin B_{12} from stores to plasma—but it merits discussion because it may have relevance in other areas. Firstly, the situation in which the serum vitamin B_{12} level is low when it is unlikely that the body stores of vitamin B_{12} are depleted; for instance in folate deficiency states and in which therapy with folic acid is followed by a rise in serum vitamin B_{12} level to normal (Mollin & Ross, 1957; Johnson *et al*, 1962; Mollin *et al*, 1962). The low serum vitamin B_{12} levels in such situations might be explicable on the grounds that folate corrects impaired release of vitamin B_{12} from stores to plasma either independently of, or in association with, folate induced correction of unduly rapid clearance of vitamin B_{12} from plasma. Secondly, and on this basis, the mechanism might be relevant to the problem of vitamin B_{12} and folate interrelationship in pernicious anaemia, the haematological response to folate being due, as suggested by Narayanan *et al* (1956) and Will *et al* (1959), to 'squeezing' of vitamin B_{12} from stores to tissues possibly by an effect on membrane transport mechanisms.

Whether the serum vitamin B_{12} or the body vitamin B_{12} have an equal role in determining the nature of erythropoiesis or whether one is more important than the other has not been established. The finding of a correlation between the serum vitamin B_{12} and body vitamin B_{12} in control subjects (Adams *et al*, 1970) and the observation that the serum vitamin B_{12} and body vitamin B_{12} decline in parallel in most cases of pernicious anaemia when treatment is withheld (Boddy & Adams, 1972) suggests that both have an equal influence. There are some indications that this may not always be so.

Treatment of patients with pernicious anaemia in relapse with 1 μg cyanocobalamin daily by injection causes marrow reversion in a few days when the body vitamin B_{12} would be little altered and this reversion is associated with changes in the serum vitamin B_{12} level (Mollin & Ross, 1953). In addition, when patients with pernicious anaemia in relapse are treated with single parenteral doses of 100 μg cyanocobalamin, erythropoiesis becomes normoblastic but reverts in about 2 weeks; not only is the nature of erythropoiesis again associated with changes in the serum vitamin B_{12} level (Mollin & Ross, 1953) but it seems unlikely that more than part of the retained dose could have been utilized if this had proceeded at the equilibrium rate of 0.1–0.2% of stores per day (Adams & Boddy, 1968, 1971; Boddy & Adams, 1968). However, if the rate of utilization of vitamin B_{12} for erythropoiesis is about 1–10 μg/day (Boddy, 1971) this would also be compatible with marrow reversion of this duration and with the minimal changes in body stores. An example of this situation occurred in Case 20 who had a relapse with megaloblastic erythropoiesis and a serum vitamin B_{12} level of 73 pg/ml while retaining about 80 μg more vitamin B_{12} than in initial relapse. From this it might be suspected that the serum vitamin B_{12} level is more immediately important than the body vitamin B_{12} in determining the nature of erythropoiesis and this suspicion is sharpened by the results in Case 2 who was found to have megaloblastic erythropoiesis with a serum vitamin B_{12} level of 134 pg/ml although the body vitamin B_{12} was in the control range at 1475 μg. It is relevant to note that this patient not only had an unusually high pretreatment body vitamin B_{12} but that the fall in serum vitamin B_{12} after treatment was unduly rapid when compared to the fall in body vitamin B_{12} (Boddy & Adams, 1972). Taken together these findings constitute evidence for the serum vitamin B_{12} being more immediately important in determining the nature of erythropoiesis than the body vitamin B_{12}. It is, of course, to be written into the case that the serum vitamin B_{12} and hence the nature of erythropoiesis are governed in turn by factors such as the release of vitamin B_{12} from stores via plasma to the erythropoietic system and the clearance of vitamin B_{12} from plasma.

The results of this and other studies emphasize that the relationship between serum vitamin B_{12} and body vitamin B_{12} is one which may vary considerably depending on a variety of factors. For this reason it is unwise to assume that the serum vitamin B_{12}, although valuable in relation to the nature of erythropoiesis, is an index of the body stores of vitamin B_{12} in the absence of knowledge of factors which are summarized as the quotient of the body vitamin B_{12} and serum vitamin B_{12}.

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Hemoglobin levels, vitamin B_{12}, and folate status in a Himalayan village[1,2]

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The difficulty of access in the high Himalayas of Nepal has hindered understanding of potential nutritional problems that may beset the inhabitants of this area. The large number of persons who live at high altitudes throughout the world and the necessity for research in such areas has recently been emphasized (1). A preliminary survey for hematologic abnormalities carried out in April 1972 by one author (WHA) among the Sherpas living at 13,000 feet near the base of Mt. Everest suggested deficiencies of either folic acid or vitamin B_{12}. This was surmised from fresh peripheral blood smears from 67 subjects, mostly school-age children, of whom 25% had definite polymorphonuclear hypersegmentation. To determine the significance of this finding, the following study was undertaken.

Subjects and methods

In October 1972, blood specimens were collected from 52 adult inhabitants of Tibetan ancestry in the Himalayan village of Lang-Tang, altitude 12,000 feet, located approximately 60 miles north of the capital of Nepal, Kathmandu. This number of specimens included almost 20% of the village population. The time of this study was during the main harvest season in the Himalayas.

Non-anticoagulated Vacutainers were used to collect serum for B_{12} and folate assays. EDTA anticoagulated specimens were used for determinations of hemoglobin, hematocrit, mean corpuscular hemoglobin concentration (MCHC), reticulocyte count, and to prepare hemolysates for red cell folate levels. *Euglena gracilis* and *Lactobacillus casei* were used for bioassays of B_{12} and folate, respectively, with red cell hemolysates for folate assay from 45 subjects prepared according to the method of Hoffbrand (2). In addition, milk samples from the yak, a bovine capable of living at extremely high altitude, were frozen and similarily assayed, the B_{12} assay being performed with and without papain digestion.

Results

The vitamin B_{12} and folate values obtained from humans are presented in Table 1. Only one subject, a 25-year-old female with a hemoglobin of 12.75 g/100 ml (#45) had a significantly low B_{12}. Information as to whether she was pregnant (a cause of low serum B_{12}) was not obtained. This subject also had a low MCHC. All serum folates were normal except for three whose levels were between 5 and 6 ng/ml (an indeterminant range (3)). Six subjects had red cell folates below 150 ng/ml, the lowest being 116 ng/ml. However, all but one of these had normal serum folate levels. The B_{12} and folate content of yak milk are presented in Table 2.

Mean hemoglobin values and one standard deviation for adult males were 16.8 ± 1.4 g/100 ml and for adult females 14.5 ± 0.7 g/100 ml. Subjects with a low MCHC or anemia as defined by the World Health Organization at sea level (3) were excluded from these calculations. Only three (6%) of 52 subjects had an MCHC below 32%. Except for one subject with severe hypochromic anemia, reticulocyte counts were normal. Hemoglobinopathies and thalassemia were not encountered (4).

Discussion

The staple foods in this village included potatoes, buckwheat, and green vegetables such as mustard greens, radishes, and nettles. Potatoes were boiled and skinned before eating. Buckwheat flour is cooked in a variety of methods, occasionally with the addition of hot peppers. The common alcoholic beverage,

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"chang," is prepared from fermented grains, and is drunk by all except the very young. Chicken, mutton, or yak meat are an uncommon but welcome addition to the diet. However, the value of these animals as a source of eggs, wool, and milk usually prevent them from being slaughtered for meat. In addition, there is an element of Buddhist cultural bias against killing of animals, although these people were not vegetarians. Milk availability fluctuates seasonally, and because of the economic advantage in selling milk to a local cheese factory for export, further curtailment of milk supply to the villagers probably occurs.

TABLE 1
Vitamin B_{12} and folate levels in Lang-Tang Village, October 1972

Subject no.	Serum B_{12}, pg/ml	Serum folate, ng/ml	Erythrocyte folate ng/ml
1	640	14.0	300
2	600	9.4	
3	600	16.5	
6	480	6.3	304
9	576	12.2	289
13	220	8.1	235
16	320	9.1	310
17	160	6.2	
18	344	5.6	206
19	240	13.8	289
20	152	7.0	182
21	416	8.4	354
22	320	8.8	
23	320	12.3	216
24	400	16.5	
25	452	8.0	
26	200	13.8	365
27	172	20.5	263
28	400	10.0	245
29	580	11.0	202
31	172	8.1	277
32	360	12.2	265
34	552	10.0	337
35	416	12.2	282
36	480	11.3	328
37	416	13.8	527
38	232	10.0	219
39	1,840	20.0	320
41	520	10.5	149
42	520	9.1	414
43	320	12.2	
44	360	15.0	324
45	128	8.4	163
46	172	11.3	180
48	288	40.0	240
49	430	12.3	342
51	320	15.0	217
52	328	9.0	119
53	208	10.2	297
54	552	7.5	328
55	344	6.7	182
56	504	5.2	145
57	760	7.1	116
58	500	7.6	294
59	300	8.1	160
60	600	7.6	242
61	552	6.7	153
62	560	10.2	177
63	452	8.4	123
64	580	7.5	250
65	552	6.4	126
66	416	5.6	183

TABLE 2
Vitamin B_{12} and folate content of yak milk

	Amount
Folate	31 μg/liter
B_{12}, plain	6,870 pg/ml
B_{12}, papain digested	7,270 pg/ml

The B_{12} content of yak milk (although the correct terminology for a female yak is "nak") is similar to cow's milk; i.e., approximately 6,000 pg/ml (5). However, the folate content was only 31 μg/liter, as compared with almost 55 μg/liter for fresh cow's milk (6). As all milk consumed by the villagers was boiled before drinking, this undoubtedly decreased the folate content further. The butterfat content, on the other hand, is 7 to 9% (7).

Despite the apparently restricted dietary status of the villagers studied, there appeared to be no evidence for significant B_{12} or folate deficiency at the time of this study. Thus, the authors' preliminary observation of neutrophile hypersegmentation noted among inhabitants living near Mt. Everest that suggested megaloblastosis was not substantiated by specific B_{12} and folate assays in a similar high altitude village. However, the former observation was made in April and the latter in October. Thus, the possibility of significant seasonal variation in availability of B_{12} and folate, especially the latter, remains to be evaluated. Nevertheless, it seems unlikely that seasonal deficiency would be an etiologic factor causing anemia in this population. Serum B_{12} levels were, with one exception, normal. The liver stores of vitamin B_{12} are considerable, and their depletion, by virtue of enterohepatic circulation (8), is slow. Indeed, the statement has been made that megaloblastic anemias due to "uncomplicated dietary deficiency of vitamin B_{12} have yet to be demonstrated in man" (9). Similarily, red cell folate levels, an indicator of tissue folate

stores, did not suggest a significant relationship to anemia, as 133 days of almost total dietary deficiency of folate are required to produce early megaloblastic changes in a normal subject (10).

Nevertheless, hemoglobin levels were considerably lower than one would expect for subjects living at that altitude (11, 12). However, only 6% had a low MCHC, indicating a minimal problem with iron deficiency, and a generally adequate level of nutrition of the population is supported by simultaneous anthropological (height versus weight) measurements made of approximately 20 young children in the same village (H. Zeigler, unpublished observations). The only gross deficiency found, and one in common to mountainous areas of Nepal in general, was that of iodine. Goiters were ubiquitous, and clinical hypothyroidism, deaf mutism, and cretinism were occasionally noted. Possibly subclinical hypothyroidism may have some bearing on the lower hemoglobin levels, but further investigation is required. It is possible that genetic factors are involved. If subsequent evidence suggests this is the case, then one must question whether "normal" hemoglobin values for man can be universally applicable, even when one considers the effect of altitude.

Summary

A survey of 52 adult inhabitants of Tibetan ancestry in a village located at 12,000 feet in the Nepal Himalayas revealed lower hemoglobin levels than that reported for populations living at similar altitude elsewhere in the world. However, serum B_{12}, serum and red cell folate, and mean corpuscular hemoglobin concentrations gave no evidence for megaloblastosis or iron deficiency as a cause of anemia. Although the role of other deficiencies such as iodine remains to be evaluated, present data do not suggest that the lower hemoglobin values found are indicative of anemia in this population.

The authors wish to express their appreciation to Dr. B. R. Baidya, Director General, and Dr. N. K. Shah, Chief Epidemiologist, His Majesty's Government of Nepal, Directorate of Health Services, for their approval and assistance of this study. The kindness of Dr. L. Paudayl, Superintendent, Central Health Laboratory, is also acknowledged. In his facility all technical procedures were performed except for the B_{12} and folate assays.

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BBA Report

BBA 21314

Methyl transfer from methyl vitamin B_{12}

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SUMMARY

The transfer of the methyl group from methyl vitamin B_{12} to thiolates and other anions has been studied. In the presence of oxidising agents, including molecular oxygen, transfer is rapid. In the absence of such reagents no methyl transfer is observed. The known mechanisms for methyl transfer in model systems are now (1) Lewis acid attack, (2) one-electron reduction and (3) one-electron oxidation. Observations on these reactions are contrasted with biological methyl transfer.

Enzyme transfer of methyl groups from methyl vitamin B_{12} is of direct importance in biological methylation of thiolates and in the generation of methyl mercury. An understanding of the second reaction has come from the study of the production of methyl mercury from methyl-B_{12} and Hg(II) cations in model systems. The reaction is a simple Lewis acid attack and occurs with other metal cations such as Tl(III)[1]. Biological transfer to thiolate, *e.g.* methionine synthesis, has been claimed[2] to be related to simple thiolate attack on methyl vitamin B_{12},

$$CH_3^- Co(III) + RS^- \longrightarrow RSCH_3 + Co(I)$$

but this observation has been questioned[3,4]. We have therefore re-examined the model reaction and made a more extensive study of methyl transfer to anions.

Solutions of $1 \cdot 10^{-4}$ M methyl cobalamin and $2.5 \cdot 10^{-2}$ mercaptoethanol in 0.1 M phosphate buffer, pH 7.8, were placed in a 1-cm absorption cell and the reaction followed by spectrophotometry. An isobestic point at 480 nm was observed as absorption fell at 520 nm, loss of methyl-B_{12}, and rose at 450–470 nm. The reaction product which absorbed at 460 nm was found to be a mixture of vitamin B_{12r}, bound to methylmercapto ethanol and its thiolate anion. The nature and stability of these complexes was established by a series of experiments using mixtures of B_{12r}, preformed by controlled potential reduction, and thiol in different molar ratios and at different pH values, following com-

plex formation by changes in the absorption and EPR spectra. The stability constants in the case of mercaptoethanol[5] are

$$B_{12r} + RSH \rightleftharpoons B_{12r}\cdot RSH \quad K_1 = 10$$
$$B_{12r} + RS^- \rightleftharpoons B_{12r}\cdot RS^- \quad K_2 = 3\cdot 10^3$$

It is known that B_{12r} is readily produced from B_{12a} under the reaction conditions. The organic product of the reaction between methyl-B_{12} and thiol was shown to be a methylated thiol by gas–liquid chromatography. That direct transfer of methyl from methyl vitamin B_{12} to the thiol had occurred was thrown in doubt by the additional observation that the reaction only proceeded in the presence of air. The effects of oxygen pressure, pH, the concentrations of the reactants, and of other added oxidising agents were therefore studied.

A detailed study of the rate of the thiolate/methyl-B_{12} reaction showed that it had the following characteristics in a steady supply of oxygen. There was (1) A lag period which was inversely dependent on thiol concentration and which could be progressively reduced by increasing pH above pH 6.0 (there is no lag at pH $>$ 8.5), by adding increasing catalytic amounts of preformed B_{12r}, or by increasing the oxygen pressure. (2) After the lag period a steady rate of disappearance of methyl vitamin B_{12} was achieved. The steady-state rate increased linearly with the oxygen pressure, methyl vitamin B_{12} concentration, added amounts of vitamin B_{12r}, and the pH but was independent of the thiol concentration. No effect on the steady state or the time lag were found on addition of trace metals. When the solutions were scrubbed free of oxygen the reaction proceeded to less than 5% completion and then stopped. The reaction could be started again by passing oxygen. Table I gives typical results.

TABLE I

RATE OF REACTION OF METHYL COBALAMIN ($1\cdot10^{-4}$ M) WITH MERCAPTOETHANOL AT 25° AND IN 0.1 M PHOSPHATE BUFFER

Thiol concn. (M)	*Ambient gas*	*pH*	*Steady-state rate**	*Time lag (min)*
$2.5\cdot10^{-2}$	N_2	7.4	0.0	
	Air	7.4	0.14	23
	O_2	7.4	0.26	17
$2.5\cdot10^{-2}$ *plus* $2\cdot10^{-6}$ M vitamin B_{12r}	O_2	7.4	0.5	2
$2.5\cdot10^{-2}$	O_2	6.4	0.5	70
		7.0	0.37	25
		7.8	0.23	5
		8.7	0.17	0
		10.5	0.05	0
$5.0\cdot10^{-2}$	O_2	7.8	0.23	0
$2.5\cdot10^{-2}$	O_2	7.8	0.23	6
$1.7\cdot10^{-2}$	O_2	7.8	0.20	25
$1.2\cdot10^{-2}$	O_2	7.8	0.25	42

*The steady-state rates in the table are quoted as the rate of fall in absorbance at 520 nm per min.

The above series of observations have much in common with those of Peel[6] on the catalysed oxidation of thiols by molecular oxygen in the presence of vitamin B_{12a}. Peel found: (1) the autooxidation produces B_{12r} (actually, as shown by us, a thiol complex of B_{12r}); (2) in the steady state the oxygen consumption is linear with time; (3) the reaction is first order in B_{12a}; (4) the reaction shows a pH dependence with an apparent pK_a of 7.5–8.0; (5) the reaction has a complex dependence on thiol concentration. The following reaction scheme explains all his results:

$$\text{Co·RSH} + O_2 \xrightarrow{k_1} \text{Co(III)} + HO_2^- + RS^\cdot$$

$$\text{Co·RS}^- + O_2 \xrightarrow{k_2} \text{Co(III)} + O_2^- + RS^\cdot$$

$$\text{Co(III)} + RSH \xrightarrow{k_3} \text{Co(II)} + RS^\cdot + H^+$$

$$\text{Co(III)} + RS^- \xrightarrow{k_4} \text{Co(II)} + RS^\cdot$$

$$RS^\cdot + RS^\cdot \xrightarrow{k_5} \text{products}$$

Putting T_{Co} as total B_{12} present, T as total thiol, using K_1 and K_2 as the formation constants of the B_{12r} complexes with thiol and thiolate, respectively, K_a as the acid dissociation constant of the thiol, and p_{O_2} as the oxygen pressure we have derived the rate expression in the steady state:

$$R = \frac{p_{O_2} \cdot T_{Co} \left\{ \dfrac{K_1 k_1}{1 + \dfrac{K_a}{[H^+]}} + \dfrac{K_2 k_2}{1 + \dfrac{[H^+]}{K_a}} \right\}}{1 + \dfrac{K_1 T}{1 + \dfrac{K_a}{[H^+]}} + \dfrac{K_2 T}{1 + \dfrac{[H^+]}{K_a}}}$$

This expression explains Peel's observations quantitatively using our values of K_1, K_2 and K_a.

The reaction of methyl-B_{12} with thiols can now be considered. The time lag is readily shown to be due to the slow establishment of a steady state in B_{12r}. This reaction step is autocatalytic, depending upon the presence of B_{12r} itself and oxygen. The initial requirement for B_{12r} is satisfied by trace impurities in methyl-B_{12} due to slight and unavoidable photolysis. The production of $RS^\cdot$ radicals from thiol by Peel's autooxidation

mechanism generates additional B_{12r} and the reaction proceeds as follows:

$$RS^{\cdot} + MeB_{12} \longrightarrow B_{12a} + Me^{\cdot} + RS^{-}$$
$$RS^{-} + B_{12a} \longrightarrow B_{12r} + RS^{\cdot}$$
$$RS^{\cdot} + Me^{\cdot} \longrightarrow RSMe$$

These reactions can be combined with the set of reactions for thiol autooxidation, shown above. In the presence of O_2, thiol and B_{12} a steady state is set up in thiol radicals and this leads to a steady state in the disappearance of methyl-B_{12}. Rate expressions for this mechanism are readily obtained and reproduced the experiment observations[5]. There is only a one-electron oxidation attack on methyl-B_{12} and no direct reaction with thiolate or thiol.

Such a direct one-electron oxidation has been demonstrated using quite different oxidising agents[7]. In acid solution and in the presence of Fe(III) ions or of $[Fe(CN)_6]^{3-}$ methyl-B_{12} has been shown to be converted rapidly to B_{12a}. Methyl radicals which are formed are readily scavenged by added chloride to give methyl chloride or by thiolates to give thio-ethers. A similar reaction occurs in the presence of redox couples such as Pt(II)/Pt(IV), Au(I)/Au(III) when the methyl group is first transferred to the metal and then to such anions as chloride[7].

We have shown now that there are three routes by which methyl groups can be transferred from methyl-B_{12} to scavenger groups. They are (a)Lewis acid attack[1], (b) one-electron reduction[12], (c) several methods of oxidation. Base attack is not known and even fails in the case of attack by cyanide. There is no evidence from model studies that direct thiolate-base attack on methyl-B_{12} occurs. It is therefore possible that biological methyl transfer[8,9] is a one-electron reaction coupled with scavenging by thiolate and it is worth looking for a radical intermediate in such methyl transfer reactions according to the scheme.

$$RS^{-} + CH_3\text{-}B_{12} + e \longrightarrow B_{12r} + CH_3SR$$
$$B_{12r} + e \longrightarrow B_{12s}$$
$$B_{12s} + \text{methyl source} \longrightarrow CH_3\text{-}B_{12}$$

TABLE II

DIFFERENTIAL ALKYL TRANSFER FROM ALKYL-B_{12} IN DIFFERENT REACTIONS

Reaction system	*Methyl-B_{12}*	*n-Propyl-B_{12}*	*Acceptor*	*Ref.*
Hg(II),Tl(III)	+	−	Metal	1
Fe(III)/Cl^-	+	+	Halide	7
$[Fe(CN)_6]^{3-}$/Cl^-	+	+	Halide	7
O_2/ thiol	+	−	Thiolate	
Pt(IV),Pt(II)/Cl^-	+	+	Halide or metal	7
Au(III),Au(I)/Cl^-	+	+	Halide or metal	7
Reduction (polarography)	+	+	Metal (Hg)	12
Enzyme	+	−	Thiolate	8,9
I_2, ICl	+	−	I^+	11

Comparison of *n*-propyl-B_{12} with methyl-B_{12} in model reactions as studied here, and in the enzyme, Table II, reveal the peculiarity that only in the reactions with Lewis acids, oxygen, and in the enzyme is *n*-propyl release very different in rate from methyl release. It could be that methyl transfer in these cases takes place in a local cage whether it is a radical reaction or not. If such a local cage reaction occurs the mechanism of methyl transfer becomes very comparable with the glycol rearrangement reaction catalysed by B_{12} enzymes[10] and both reactions have much in common with the radical-cage mechanism proposed for the Stephen's rearrangement[13].

Note. Since this paper was submitted it has been shown that vitamin B_{12r} is an intermediate in the reactions of the enzyme vitamin B_{12}-dependent methionine synthetase[14].

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SPECULATIVE ARTICLES

It is proposed occasionally to publish articles of a speculative or interpretive nature. These articles should be of not more than ten pages and should indicate either a new interpretation of data, a new idea, or indicate a different manner of looking at present data. They will have a heading "Speculative Article".

G. A. KERKUT

SPECULATIVE ARTICLE

THE BIOCHEMICAL ROLE OF VITAMIN-B_{12}

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Abstract—1. The active form of vitamin-B_{12} which takes part in biological reactions is either 5′-deoxyadenosyl-B_{12} or methyl-B_{12}.

2. Both these coenzymes contain a cobalt—carbon bond.

3. It is proposed that these coenzymes owe their biochemical role to the property of the cobalt—carbon bond to dissociate into two reactive species, a highly reduced cobalt and a carbonium ion.

INTRODUCTION

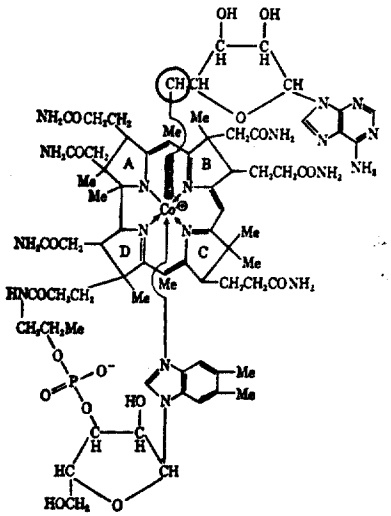
METAL ions play an important role in biological systems. Iron, for example, has been exploited by nature in at least two major ways. It can act as oxidation–reduction agent through a transformation of the type $Fe^{2+} \rightleftharpoons Fe^{3+}$; this property of iron is utilized in the reactions of cytochromes. Another feature of the Fe^{2+} is its ability to be converted from an ionic ferrous state, as exists in $FeCl_2$, to a covalent ferrous state in which iron is attached to six negatively charged ligands as in $K_4Fe(CN)_6$. The conversion of hemoglobin into oxyhemoglobin involves a change from an ionic ferrous to a covalent ferrous state.

Some other metal ions like Mn^{2+}, Mg^{2+} and Ca^{2+} act as cations to complex with negatively charged substrates and negatively charged regions of the enzymes thus profoundly modifying the properties of the ligands.

The present paper proposes that the cobalt atom is involved in biological reactions because of its unique property to form a cobalt—carbon bond. Such a bond is present in two major coenzymes 5′-deoxyadenosyl-B_{12} (Structure 1) and methyl-B_{12} (Structure 1a), derived from vitamin-B_{12}. Both these coenzymes owe

their biochemical role to the property of the cobalt—carbon bond to dissociate into two highly reactive species, a highly reduced cobalt and a carbonium ion.

Vitamin-B_{12} can be converted both enzymically and chemically into two related compounds, 5′-deoxyadenosyl-B_{12} (Structure 1) and methyl-B_{12} (Structure 1a) which then act as coenzymes in a variety of biological transformations. There is a unique property of the cobalt atom which has been ingeniously exploited by nature for carrying out diverse biochemical reactions. It is first necessary to discuss the mechanism of those enzymic reactions which require the participation of 5′-deoxyadenosyl-B_{12} (for a review see Barker, 1967).

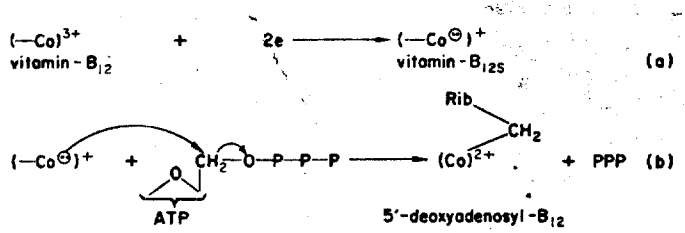


STRUCTURE 1. 5′-Deoxyadenosyl-B_{12}.
STRUCTURE 1a. Methyl-B_{12}; the heavy bond in the above structure is replaced by a methyl group.

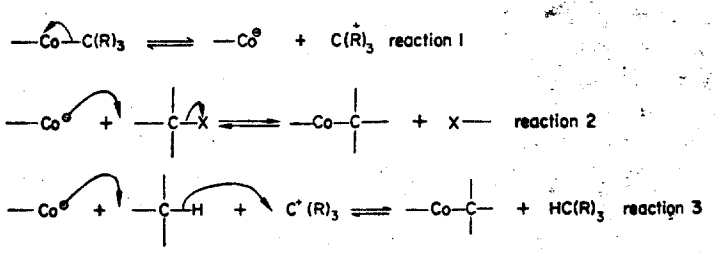
In a recent report it has been shown (Frey & Abeles, 1966) that the hydrogens attached to carbon atom 5′ of the deoxyadenosyl moiety of the coenzyme (Structure 1, the 5′-methylene group under discussion is encircled in the formula) are involved in the biochemical reactions. This and related observations have prompted us to propose the chemical role of the coenzyme in enzymic reactions. It is suggested that the catalytic role of the 5′-deoxyadenosyl-B_{12} coenzyme is associated with three fundamental properties of this cobalt atom.

(a) The cobalt—carbon bond in the coenzyme is so polarized that it can be dissociated into two charged species, a reduced cobalt atom and a positively charged carbon atom; this is shown in reaction 1 (Scheme 1). This property of the cobalt—carbon bond can be deduced from the mode of chemical (Johnson *et al.*, 1963) as well as biochemical (Peterkofsky & Weissbach, 1964) synthesis of 5′-deoxyadenosyl-B_{12} coenzyme (Structure 1). This coenzyme (Structure 1) is biosynthesized

through the involvement of vitamin-B_{12}, a reducing agent and ATP, the overall transformation is believed to occur in two steps as shown below in equations (a) and (b). The first step results in the reduction of cobalt atom from a valency state



of three to a highly reduced monovalent species (equation a). The two electrons added in this reaction are shown by two dots (such a species is called B_{12s}). This pair of electrons is then utilized in the next step to form a carbon—cobalt bond and the elimination of tripolyphosphate. Analogous reactions in other areas of organic chemistry will require that the cobalt atom in 5′-deoxyadenosyl-B_{12} coenzyme should be in a valence state of 2. A formal reversal of reaction shown by equation (b) would allow one to deduce the property of the cobalt—carbon bond we have



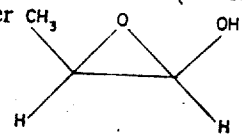
SCHEME 1.

advocated in reaction 1, Scheme 1: that is, the cobalt—carbon bond in the coenzyme has the tendency to be cleaved so that the pair of electrons forming the bond are retained by cobalt, thus converting it into a highly reduced monovalent form, while the 5′-carbon atom becomes positively charged (equation c). For the sake of convenience in the subsequent representation of the coenzymes and related intermediates we have not shown the charge on the cobalt atom.

(b) The reduced cobalt species obtained in reaction 1 (Scheme 1) can participate in nucleophilic displacements at carbon atoms containing good leaving groups. This is shown in reaction 2 (Scheme 1).

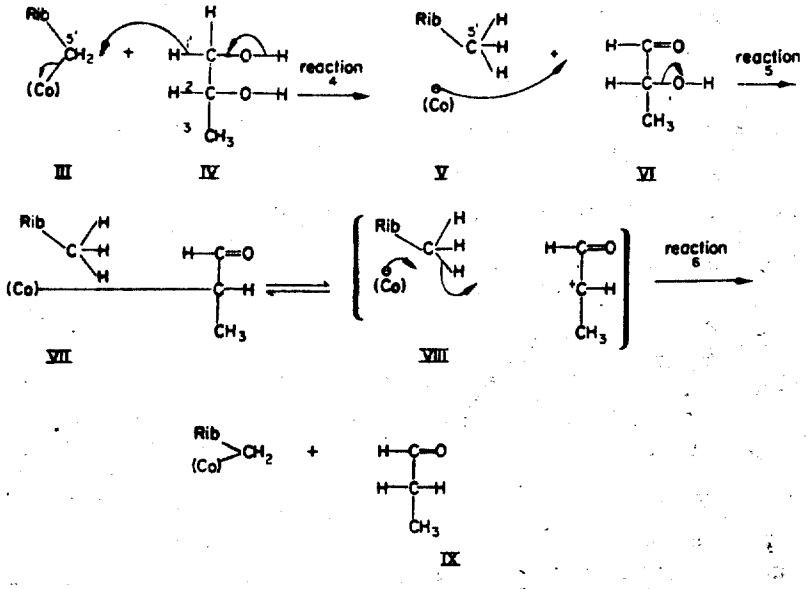
(c) Finally, it is suggested that the cobalt species of the type II (Scheme 1) can displace a hydride from a saturated carbon atom in the presence of a suitable hydride acceptor, as shown by reaction 3 (Scheme 1). In reaction 3 the formation of a cobalt—carbon bond and the transfer of a hydrogen to a carbonium ion has occurred through a "push–pull" concerted process. In the light of these reactions we shall now consider the mechanisms of three selected enzymic reactions.

Dioldehydrase catalysed conversion of 1,2-propanediol into propionaldehyde

Dioldehydrase catalyses the conversion of 1,3-propanediol into propionaldehyde (IV→IX, Scheme 2). It is proposed that this conversion occurs through a hydride transfer from C-1 of propanediol to C-5′ of the cobamide coenzyme, thus resulting in the formation of a methyl group and the reduced species of Co (reaction 4, Scheme 2, cf. reversal of reaction 3 in Scheme 1). The subsequent regeneration of the coenzyme and the formation of propionaldehyde is indicated by reactions 5 and 6 (Scheme 2). The C—H bonds which are attached to a methyl group of the type shown in Structure VIII (Scheme 2) are normally supposed to be unreactive thus making it difficult to involve them in reactions. It is, however, to be noted that in this particular case the methyl hydrogen atoms would be located between two reactive species, a highly reduced cobalt atom and a carbonium ion thus enabling them to function in hydrogen transfer reactions. Although other pathways with minor modifications for the formation of the intermediate VII or VIII from III+IV are feasible, however, all these pathways according to the theory under discussion must result in the formation of a methyl group at C-5′ during the enzymic reaction. For example, the ^{18}O experiments of Retey *et al.*, (1966) may be rationalized by assuming that in reaction 5 (Scheme 2) instead of the intermediate (VI, Scheme 2) its isomer  is involved.

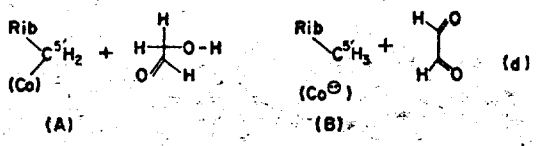
The mechanism proposed in Scheme 2 would require that during the enzymic reaction the two hydrogen atoms attached to C-5′ of the deoxyadenosyl moiety and one of the two hydrogen atoms at C-1 of the propanediol should become indistinguishable. This is borne out by the work of Frey & Abeles (1966). It has been shown that when a catalytic amount of cobamide coenzyme labelled equally with tritium at both the hydrogen atoms attached to C-5′ of the deoxyadenosyl moiety is used in the dioldehydrase reaction the entire radioactivity of the coenzyme is found in propionaldehyde (Frey & Abeles, 1966). This observation cannot be reconciled with the known principles of enzymology unless one assumes that at some stage in the enzymic reaction the C-5′ methylene group is converted into a methyl group (Barker, 1967), thus rendering the two C-5′ hydrogens sterically equal. Also, it has been shown that in the dioldehydrase reaction when non-radioactive coenzyme is incubated with 1,2-propanediol-1-^3H, the recovered coenzyme is radioactive (Frey & Abeles, 1966). This incorporation of radioactivity

is readily explained by the mechanism proposed in Scheme 2. Furthermore, this mechanism (Scheme 2) allows one to predict that if in the dioldehydrase reaction 1,2-propanediol-1-^{3}H* is incubated with non-radioactive coenzyme so that the molar ratio of each is 1 : 1, then the isolated propionaldehyde should contain only a third of the radioactivity of the parent diol (or less because of the isotope effect which is likely to be associated in the hydrogen transfer in reaction 6, Scheme 2), the remaining being incorporated into the coenzyme.



SCHEME 2.

Another important observation which can be satisfactorily explained by the general mechanism under discussion involves the dioldehydrase-catalysed reaction of glycolaldehyde and cobamide coenzyme. This reaction is accompanied by the formation of glyoxal and the inactivation of the enzyme–coenzyme complex. This may be explained as follows:



* It is to be noted that only *one of the two* hydrogen atoms at C_1 of propanediol takes part in the reaction. It is implied that in the conversion the substrate contains only that hydrogen stereospecifically labelled with tritium which participates in the enzymic reaction.

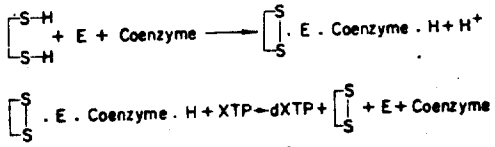
The first step is the transfer of a hydride ion from the primary alcohol group (cf. reaction IV, Scheme 2) thus resulting in the formation of glyoxal and the coenzyme intermediate (B). Since the product, glyoxal, cannot participate in any further reaction the coenzyme (A) cannot be regenerated. This mechanism would predict that the inactivation of the enzyme–coenzyme complex should be attended by the formation of 5-deoxyadenosine. This *seems* to be the case (Wagner *et al.*, 1966).

Ribonucleotide reductase conversion of ribonucleoside triphosphate into 2′-deoxyribonucleoside triphosphate

Cobamide-coenzyme-dependent ribonucleotide reductase catalyses the reaction shown below.

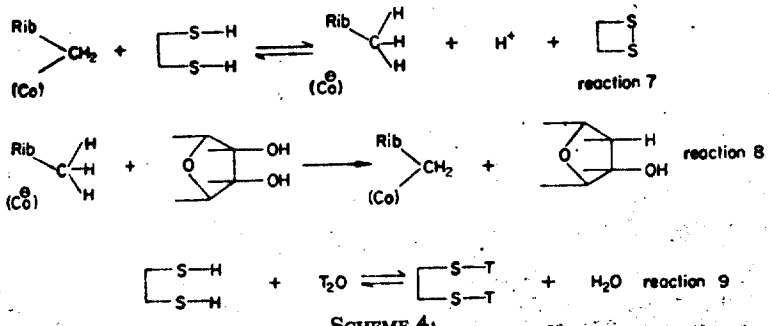
$$XTP + \text{dihydrolipoate} \rightarrow dXTP + \text{lipoate}.$$

In an elegant work Abeles & Beck (1967) have recently proposed a two-step sequence shown below for this reaction. The above reaction may be elaborated in terms of a



SCHEME 3.

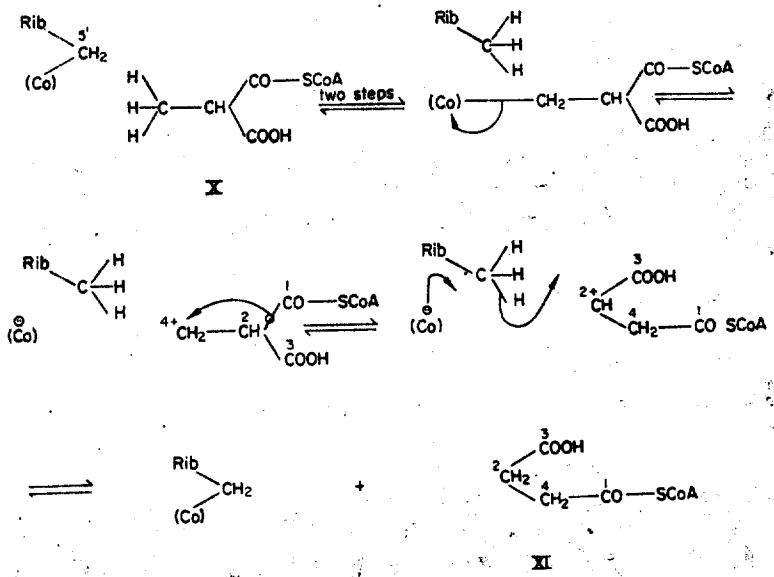
detailed mechanism outlined in Scheme 4. Reaction 7 (Scheme 4) indicates the transfer of a hydride ion from dihydrolipoate to the coenzyme thus resulting in the formation of a methyl group and $CO^{\ominus}$. The subsequent formation of the deoxy derivative is shown by reaction 8 (Scheme 4) and occurs in three steps through the combination of reactions of the types 2 and 3 (Scheme 1). Consistent with the proposed mechanism (Scheme 4) is the observation (Abeles & Beck, 1967) that when a ribonucleotide reductase catalysed reaction is carried out with non-radioactive coenzyme in the presence of T_2O the recovered coenzyme contains two atoms of tritium at C-5′. Again, this incorporation of radioactivity in both the C-5′ hydrogens of the coenzyme strongly suggests the intermediacy of the C-5′ methyl group during the enzyme reaction. The involvement of reactions 7 and 9 (Scheme 4)



SCHEME 4.

would satisfactorily account for the complete equilibration of the two hydrogens at C-5′ of the deoxyadenosyl moiety with those of water.

The enzyme methylmalonyl–CoA mutase catalyses the conversion of methylmalonyl-CoA (X) into succinyl-CoA (XI). A mechanistic proposal based on the ideas developed above is outlined in Scheme 5 (the first step of this scheme represents the combination of reactions 1 and 3 of Scheme 1). Once again the main feature of the mechanism is the involvement of a 5′-methyl intermediate* and a carbonium ion. Similar mechanisms can be envisaged for glutamate isomerase and related reactions. Our suggestion of the participation of carbonium ions offers a unified concept to explain the mechanisms of all known cobamide-dependent enzymic reactions.



SCHEME 5.

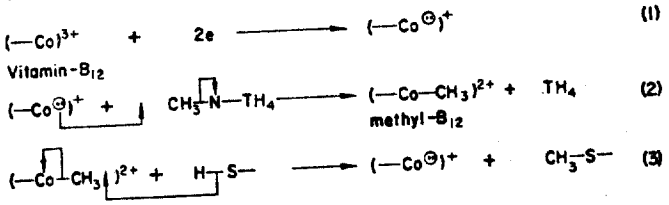
Methionine biosynthesis

One of the reactions requiring the participation of methyl-B_{12} coenzyme (Structure 1a) is the conversion of homocysteine into methionine, the reaction proceeds according to the equation:

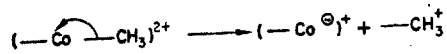
5-methyltetrahydrofolate (CH_3-N-TH_4) + homocysteine (H-S—) $\xrightarrow[\text{and a reducing system}]{\text{S-adenosylmethionine, } B_{12}}$ methionine + tetrahydrofolate.

* The first proposal for the involvement of a methyl group in the methylmalonyl-CoA mutase reaction was made by Ingraham L. L. (1964) *Ann. N.Y. Acad. Sci.* **112**, 713; however, the underlying principle of this mechanism is different from the one developed in the present communication.

Recent evidence suggests (Barker, 1967) that the first step in the above conversion is the transfer of a methyl group from 5-methyltetrahydrofolate to the cobalt atom of vitamin-B_{12} to furnish methyl-B_{12}. This reaction occurs in the presence of a reducing agent and may be envisaged to take place in two steps as shown by equations (1) and (2).



The first step (equation 1) is the transfer of two electrons to convert the cobalt atom from a trivalent state in the vitamin into a monovalent species. The pair of electrons are then utilized in the next step for the formation of a new carbon—cobalt bond to give methyl-B_{12} (equation 2), the cobalt atom in the coenzyme therefore must have a valency of two. Once again the cobalt—carbon bond in methyl-B_{12}, like the analogous bond in 5′-deoxyadenosyl-B_{12}, has the potential to be polarized so that the pair of electrons forming this bond can be transferred to the cobalt atom thus leaving the methyl carbon positively charged, i.e.



This polarization facilitates the final step of the reaction which involves the formation of a C—S bond and the regeneration of a reduced species of cobalt (equation 3). This analysis of the overall reaction would allow one to predict that the reducing agent should only be required in the first cycle, since the valency state of the cobalt atom at the end of the reaction sequence (equation 3) is suitable for its involvement in the crucial step of the reaction shown by equation (2).

To summarize, a mechanism for the cobamide-dependent enzymic reactions is proposed. It is suggested that during the enzymic reaction the cobamide coenzyme (Structure 1) suffers a reductive cleavage between the cobalt atom and the carbon atom 5′ of the deoxyadenosyl moiety thus resulting in the formation of a 5′-methyl group and $CO^{\ominus}$. In the subsequent reaction $Co^{\ominus}$ makes a bond with the substrate thus facilitating the formation of a carbonium ion. Finally, the neutralization of the parent or rearranged carbonium ion by a hydride transfer from the 5′-methyl group results in the formation of the product and the regeneration of the coenzyme.

Note added in proof—A mechanism similar to the one proposed above has independently been considered [HOGENKAMP H. P. C. (1968) *A. Rev. Biochem.* 37, 225] for the ribonucleotide reductase reaction.

Acknowledgements—My interest in this problem was stimulated through the judicious choice of papers for the *Journal Club—1967* by my colleagues, Dr. D. C. Wilton, Dr. P. F. Hunt and Mr. A. H. Rahimtula.

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Isolation of Gastric Vitamin B_{12}-binding Proteins Using Affinity Chromatography

II. PURIFICATION AND PROPERTIES OF HOG INTRINSIC FACTOR AND HOG NONINTRINSIC FACTOR*

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SUMMARY

Two vitamin B_{12}-binding proteins, designated hog intrinsic factor and hog nonintrinsic factor, have been isolated from hog gastric mucosa. Affinity chromatography on vitamin B_{12}-Sepharose resulted in the removal of the bulk of protein present in a crude extract of hog gastric mucosa. The two vitamin B_{12}-binding proteins were separated subsequently by a method of "selective" affinity chromatography with an affinity adsorbent containing covalently bound derivatives of vitamin B_{12} that lack the nucleotide portion of the native vitamin. Under appropriate conditions hog nonintrinsic factor was adsorbed to a column of this material, while hog intrinsic factor was not. After additional purification both proteins were isolated in homogeneous form based on polyacrylamide disc gel electrophoresis, sedimentation equilibrium ultracentrifugation, and sodium dodecyl sulfate polyacrylamide gel electrophoresis. Hog intrinsic factor (24 μg) corrected vitamin B_{12} malabsorption when given to a patient with pernicious anemia, while hog nonintrinsic factor (49 μg) had no effect.

Hog intrinsic factor binds 30.3 μg of vitamin B_{12} per mg of protein. Molecular weight values of 52,300 to 58,600 were obtained by sedimentation equilibrium ultracentrifugation and amino acid and carbohydrate analysis. The protein contains 17.5% carbohydrate which accounts for the elevated molecular weight values (66,000 to 75,000) obtained by sodium dodecyl sulfate polyacrylamide gel electrophoresis and gel filtration. In the presence of vitamin B_{12} hog intrinsic factor aggregates to form dimers and higher molecular weight oligomers.

Hog nonintrinsic factor binds 25.1 μg of vitamin B_{12} per mg of protein. Molecular weight values of 61,000 to 66,000 were obtained by sedimentation equilibrium ultracentrifugation and amino acid and carbohydrate analysis. The protein contains 35.5% carbohydrate which accounts for the elevated molecular weight values (100,000 to 130,000) obtained by sodium dodecyl sulfate polyacrylamide gel electrophoresis and gel filtration. Hog nonintrinsic factor retains its monomeric form in the presence of vitamin B_{12} under conditions in which hog intrinsic factor aggregates.

Hog intrinsic factor and hog nonintrinsic factor differ significantly from each other in their amino acid and carbohydrate composition. These differences, together with differences in other parameters, demonstrate that these two proteins are distinct and separate species.

Crude extracts of hog gastric mucosa have a vitamin B_{12}-binding activity of approximately 0.05 μg of vitamin B_{12} bound per mg of protein, and these extracts also contain intrinsic factor activity since approximately 60 mg of dry material are able to correct vitamin B_{12} malabsorption by patients with pernicious anemia as judged by the results of Schilling tests (1). All of the vitamin B_{12}-binding activity in these crude extracts is not attributable to hog IF,[1] however, for a second vitamin B_{12}-binding protein is present that lacks IF activity in Schilling tests (1, 2). This second vitamin B_{12}-binding protein has been referred to as hog NIF.

Studies using extracts of hog gastric mucosa or partially purified preparations of these two proteins are somewhat difficult to interpret, but they suggest that hog IF and hog NIF also differ from each other in other ways. These differences include: (*a*) anti-IF antibody obtained from the serum of certain patients with pernicious anemia blocks some of the vitamin B_{12}-binding activity present in crude extracts of hog gastric mucosa. Irvine (3) has demonstrated that the amount of anti-IF antibody-mediated decrease in vitamin B_{12}-binding activity is a more reliable guide to the Schilling test IF activity of crude hog gastric mucosal preparations than is the level of total vitamin B_{12}-binding activity. This observation suggests that the vitamin B_{12}-binding ability of hog IF is blocked by the antibody, while that of hog NIF is not. (*b*) Sera from rabbits immunized with hog leukocyte extracts contain antibodies which appear to bind to hog NIF but not to hog IF (4). (*c*) Hog IF appears to facilitate

* This work was supported by Grants AM 16668, AM 10550, and HE 00022 and Special Research Fellowship AM 51261 from the National Institutes of Health.

[1] The abbreviations used are: IF, intrinsic factor vitamin B_{12}-binding protein; NIF, nonintrinsic factor vitamin B_{12}-binding protein; pseudo-vitamin B_{12}, α-(adenyl)-cobamide cyanide.

dependent vitamin B_{12} binding by guinea pig ileal homogenates, while hog NIF does not (5). (*d*) Hog NIF appears to facilitate calcium-dependent vitamin B_{12} binding by rat liver homogenates, while hog IF does not (5). (*e*) Certain structural analogs of vitamin B_{12} are bound by hog NIF to a greater extent than they are by hog IF (1).

Numerous attempts have been made to isolate hog IF and hog NIF in homogeneous form (6). Holdsworth (2) and Ellenbogen *et al.* (1, 7) have isolated vitamin B_{12}-binding proteins from hog gastric mucosa in highly purified form and were able to separate the vitamin B_{12}-binding protein into two fractions using ion exchange chromatography. In both studies one fraction contained IF based on Schilling tests, while the second fraction did not. Immunologic and ileal and liver homogenate binding studies were not performed, but Ellenbogen *et al.* (1) did demonstrate that their IF active fraction bound only 10% as much pseudo-vitamin B_{12} as native vitamin B_{12}, while their IF inactive fraction bound 90% as much pseudo-vitamin B_{12} as native vitamin B_{12}.

Ellenbogen *et al.* (1, 7) observed a molecular weight of approximately 50,000 for both of their protein fractions in the absence of vitamin B_{12} using sedimentation velocity ultracentrifugation. In the presence of vitamin B_{12}, molecular weight values of 100,000 were obtained for both fractions suggesting that the protein in both fractions dimerized in the presence of the vitamin. Holdsworth (2) also observed molecular weight values of approximately 50,000 for both of his protein fractions, but he did not observe any significant increase when vitamin B_{12} was present. Neither Ellenbogen *et al.* nor Holdsworth were able to detect any significant differences in the amino acid compositions of their IF active and IF inactive fractions. The same type of carbohydrate residues were observed in both fractions. Ellenbogen *et al.* did note that their IF active fraction contained twice as much galactose as their IF inactive fraction, but this difference was not observed by Holdsworth who noted that hog IF contained approximately 30% less of each type of carbohydrate residue when compared with hog NIF.

Thus immunologic studies, ileal and liver homogenate binding studies, and analog binding studies all suggest that hog IF and hog NIF are structurally dissimilar. On the other hand, studies of molecular weight, protein aggregation, and amino acid and carbohydrate composition have failed to reveal any major and consistent differences between hog IF and hog NIF. It is thus unclear whether hog NIF is (*a*) a zymogen-like precursor of hog IF, (*b*) a limited degradation product of hog IF, or (*c*) structurally unrelated to hog IF.

We have previously reported (8) that the hog gastric mucosal vitamin B_{12}-binding proteins can be isolated in high yield using affinity chromatography on vitamin B_{12}-Sepharose which is prepared by covalently attaching monocarboxylic acid derivatives of vitamin B_{12} to a substituted Sepharose using a carbodiimide. Analysis of the vitamin B_{12}-binding protein obtained after affinity chromatography revealed that only 30% of the vitamin B_{12}-binding protein is IF based on assays using anti-IF antibody obtained from the serum of a pernicious anemia patient. Attempts to separate hog IF from hog NIF using ion exchange chromatography have been unsuccessful. This difficulty has been circumvented, however, and hog IF and hog NIF have been resolved using a method of "selective" affinity chromatography in which carboxylic acid derivatives of vitamin B_{12} that lack the nucleotide portion of the vitamin are covalently coupled to Sepharose. Under appropriate conditions hog NIF is adsorbed to a column of this material, while hog IF is not. This report is concerned with the isolation, separation, and properties of hog IF and hog NIF.

EXPERIMENTAL PROCEDURES

Materials

Hog intrinsic factor concentrate, type S, 80 mesh, Lot 48744-414, was obtained from Lederle. This material is a crude water extract of hog pyloric mucosa that was spray-dried without additional purification. We have stored this material in the dry state at 4° for up to 1 year without noting any loss of vitamin B_{12}-binding activity. All other materials were obtained as described in the accompanying paper in this series (9).

Methods

Preparation of Sepharose-bound Derivatives of Vitamin B_{12} Lacking the Nucleotide—Vitamin B_{12}, 10.0 g, containing 2 μCi of [^{57}Co]vitamin B_{12}, was dissolved in 500 ml of 11 N HCl and incubated at 70° for 1 hour in the dark. The solution was subsequently lyophilized, dissolved in 500 ml of H_2O, and lyophilized again. The dry material was dissolved in water to give a concentration of vitamin B_{12} derivative of 25.0 mg per ml and stored at −20°.

These derivatives of vitamin B_{12} were covalently attached to 3,3′-diaminodipropylamine-substituted Sepharose at pH 5.6 using 1-ethyl-3-(3-diethylaminopropyl)-carbodiimide. The concentration of vitamin B_{12} derivatives in the coupling reaction prior to the addition of the carbodiimide was 12.5 mg per ml. The coupling reaction and subsequent washing and storage of the Sepharose substituted with vitamin B_{12} derivatives were performed under the same conditions used for the preparation of vitamin B_{12}-Sepharose (8). The washed Sepharose containing the 11 N HCl hydrolysis derivatives of vitamin B_{12} had a vitamin B_{12} derivative content of 0.22 μmole per ml of packed Sepharose based on measurements of radioactivity.

Paper chromatography of vitamin B_{12} and its derivatives was performed as previously described (8). The amount of individual components was determined by cutting out individual spots and counting them in a Packard γ scintillation counter.

All other methods were performed as described in the accompanying paper in this series (9).

Purification of Hog IF and Hog NIF

Step 1: Preparation of Hog Gastric Mucosal Extract—All procedures were performed at 4° unless specifically noted. Hog intrinsic factor concentrate, 50 g, was added to 1.0 liter of 0.1 M Tris-acetate, pH 9.2, and stirred for 30 min. The suspension was centrifuged at 20,000 × *g* for 30 min, and the turbid supernatant was decanted. The supernatant was filtered with vacuum suction through Celite using a Buchner funnel containing a coarse scintered glass disc. The filtrate was centrifuged at 20,000 × *g* for 30 min, and the supernatant was immediately subjected to affinity chromatography on vitamin B_{12}-Sepharose.

Step 2: Affinity Chromatography on Vitamin B_{12}-Sepharose—A column (2.5 × 7.5 cm) of vitamin B_{12}-Sepharose containing 12.0 mg of covalently bound vitamin B_{12} was prepared and washed with 200 ml of 0.1 M glycine-NaOH, pH 10.0, followed by 100 ml of 0.1 M potassium phosphate, pH 7.5, immediately prior to the sample application to remove any hydrolyzed vitamin B_{12}. The flow rate was 100 ml per hour. After the entire sample had passed onto the column, the column was washed with 25 ml of 0.1 M potassium phosphate, pH 7.5. The first 900 ml of effluent were collected in their entirety. The column was

TABLE I

Affinity chromatography of hog gastric vitamin B_{12}-binding proteins

Item	Volume	Vitamin B_{12}-binding activity			Protein		Flow rate
	ml	μg/ml	total μg	% IF	mg/ml	total mg	ml/hr
Hog gastric mucosal extract applied to vitamin B_{12}-Sepharose	875	2.99	2620	20.6	27.0	23,600	100
Initial vitamin B_{12}-Sepharose effluent	900	0.0042	3.36	76.2	25.0	22,500	
Further elutions of vitamin B_{12}-Sepharose							
1. 0.1 M potassium phosphate, pH 7.5	500	0.00034	0.17	64.7	0.87	435	150
2. 0.1 M glycine-NaOH, pH 10.0, 0.1 M glucose, 1.0 M NaCl	1,000	0.00000	0.00		0.03	30	150
3. 0.1 M potassium phosphate, pH 7.5	500	0.00078	0.39	59.7	0.02	10	150
4. 0.1 M potassium phosphate, pH 7.5, 7.5 M guanidine-HCl							
a. Initial eluate	65	32.8	2130	24.4	2.68	174	40
b. Eluted 17 hours after 4a	65	0.08	5.2	27.6	0.00	0.00	40

then eluted as follows: (*a*) 500 ml of 0.1 M potassium phosphate, pH 7.5; (*b*) 1000 ml of 0.1 M glycine-NaOH, pH 10.0, containing 0.1 M glucose and 1.0 M NaCl; and (*c*) 500 ml of 0.1 M potassium phosphate, pH 7.5. The fourth elution solution consisted of 0.1 M potassium phosphate, pH 7.5, containing 7.5 M guanidine HCl. When 10 ml of this solution had passed through the column, flow was stopped. After 1 hour an additional 55 ml of eluate were collected, pooled with the first 10 ml, and designated as Eluate 4a. Flow was stopped for an additional 17 hours and then an additional 65 ml of eluate were collected and designated as Eluate 4b.

The starting material, initial column effluent, and each of the column eluates were assayed for vitamin B_{12}-binding activity, IF activity, and protein content. The results are summarized in Table I.

Eluate 4a was dialyzed against 6.0 liters of distilled water for 24 hours with dialysate changes at 4 and 16 hours. The sample was concentrated with an Amicon ultrafiltrator equipped with a Diaflo UM-10 membrane and centrifuged at 20,000 × *g* for 10 min. The supernatant, 8.5 ml, was decanted and 1.10 ml of 1.0 M sodium acetate, pH 5.0, and 1.47 ml of 7.5 M guanidine HCl were added. The sample was brought to room temperature and immediately subjected to "selective" affinity chromatography.

Step 3: "Selective" Affinity Chromatography—A column (2.5 × 18 cm) of the substituted Sepharose containing 0.30 mg per ml of covalently bound derivatives of vitamin B_{12} that lacked the nucleotide portion of the vitamin was prepared at room temperature. The column was washed with 300 ml of 0.1 M glycine-NaOH, pH 10.0, 120 ml of 7.5 M guanidine HCl containing 0.1 M sodium acetate, pH 5.0, and finally with 300 ml of 1.0 M guanidine HCl containing 0.1 M sodium acetate, pH 5.0. The sample was applied by gravity at an approximate pressure of 40 cm of H_2O. The flow rate was 50 ml per hour. The column was eluted with 200 ml of 1.0 M guanidine HCl containing 0.1 M sodium acetate, pH 5.0, followed by 130 ml of 7.5 M guanidine HCl containing 0.1 M sodium acetate, pH 5.0. Nine-milliliter fractions were collected and assayed for vitamin B_{12}-binding activity, IF activity, and A_{280}. The results are presented in Fig. 1.

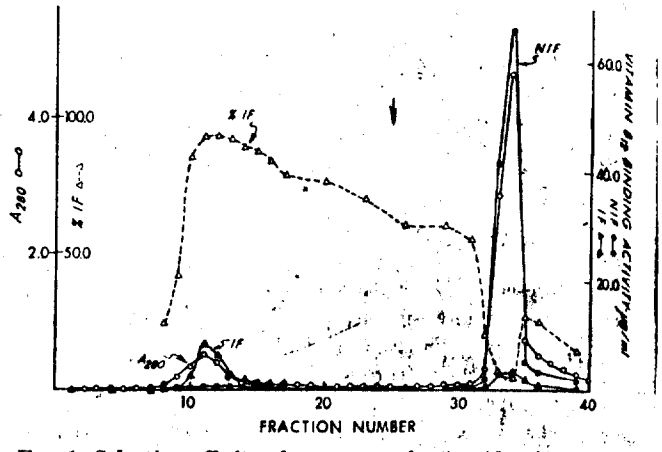

FIG. 1. Selective affinity chromatography (purification Step 3) of hog IF and hog NIF. A column (2.5 × 18 cm) of substituted Sepharose containing covalently bound derivatives of vitamin B_{12} that lacked the nucleotide portion of the vitamin was prepared at room temperature and equilibrated with 0.1 M sodium acetate, pH 5.0, containing 1.0 M guanidine HCl. The sample (from purification Step 2) consisted of a mixture of hog IF and hog NIF in 11.0 ml of equilibrating buffer. The column was eluted initially with equilibrating buffer followed, as indicated by the position of the *arrow*, by 0.1 M sodium acetate, pH 5.0, containing 7.5 M guanidine HCl. The flow rate was 50 ml per hour and 9.0-ml fractions were collected. ○, A_{280}; △, percentage of vitamin B_{12}-binding activity attributable to hog IF; ▲, hog IF; ●, hog NIF.

Further Purification of Hog IF

Repeat Affinity Chromatography on Vitamin B_{12}-Sepharose—Fractions 11 to 13 from Step 3 were pooled and dialyzed for 24 hours against 2.0 liters of 0.05 M potassium phosphate, pH 7.5, containing 0.75 M NaCl with dialysate changes at 4 and 16 hours. The sample was applied to a column (0.9 × 5.0 cm) of vitamin B_{12}-Sepharose that contained 1.2 mg of covalently bound vitamin B_{12}. The column was eluted with: (*a*) 100 ml of 0.1 M glycine-NaOH, pH 10.0, containing 0.1 M glucose and 1.0 M NaCl; (*b*) 60 ml of 0.1 M potassium phosphate, pH 7.5; and (*c*) 9.9 ml of 7.5 M guanidine HCl containing 0.1 M potassium phosphate, pH 7.5. Eluate 3 contained 121.8 μg of vitamin B_{12}-binding activity and 97.3% of this activity was blocked by prior incubation with anti-IF antibody. This material is hereafter referred to as hog IF.

Further Purification of Hog NIF

Chromatography on Sephadex G-150—Fractions 33 and 34 from Step 3 were pooled and dialyzed against 2.0 liters of 0.05 M potassium phosphate, pH 7.5, containing 0.75 M NaCl for 24

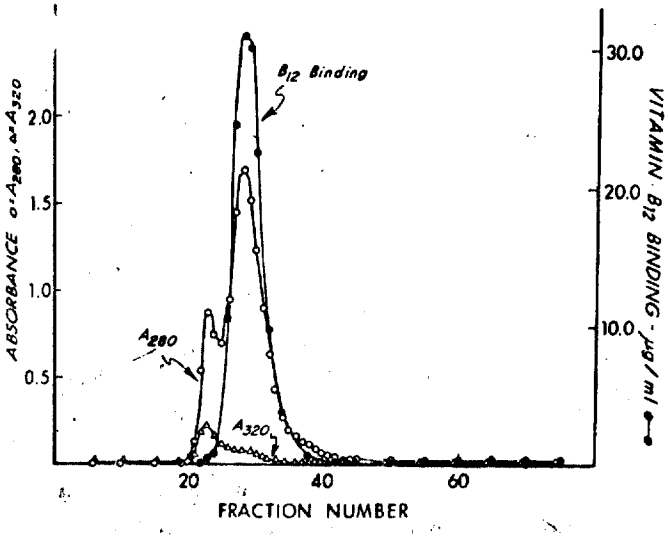

FIG. 2. Sephadex G-150 chromatography of hog NIF. The material present in Fractions 33 and 34 from selective affinity chromatography (purification Step 3) was applied to a column (2.0 × 95 cm) of Sephadex G-150 equilibrated with 0.05 M potassium phosphate, pH 7.5, containing 0.75 M NaCl. The sample volume was 6.0 ml, and 4.0-ml fractions were collected. Appropriate fractions were assayed for: ○, A_{280}; △, A_{320}; and ●, vitamin B_{12}-binding ability.

hours with dialysate changes at 4 and 16 hours. The sample was concentrated to 6.0 ml as described in Step 2 and centrifuged 900 × *g* for 10 min. The supernatant was applied to a n (2.0 × 90 cm) of Sephadex G-150, fine grade, that had been equilibrated with 0.05 M potassium phosphate, pH 7.5, 0.75 M NaCl. The flow rate was 20 ml per hour, and 4.0-ml fractions were collected. Appropriate fractions were assayed for vitamin B_{12}-binding activity, A_{280}, and A_{320}. The results are presented in Fig. 2.

Chromatography on 3,3'-Diaminodipropylamine-substituted Sepharose—Fractions 27 to 32 from Sephadex G-150 chromatography were pooled and centrifuged at 20,000 × *g* for 10 min. The supernatant was applied to a column (2.5 × 5.0 cm) of 3,3'-diaminodipropylamine-substituted Sepharose that was equilibrated with 0.05 M potassium phosphate, pH 7.5, containing 0.75 M NaCl. The column was eluted with this same solution. The first 44 ml of effluent contained 84% of the vitamin B_{12}-binding activity applied and were subjected to repeat affinity chromatography on vitamin B_{12}-Sepharose.

Repeat Affinity Chromatography on Vitamin B_{12}-Sepharose—A column (2.5 × 2.0 cm) of vitamin B_{12}-Sepharose containing 3.5 μg of covalently bound vitamin B_{12} was prepared as described above, and the sample from the preceding step was applied at a flow rate of 100 ml per hour. The column was eluted with: (*a*) 100 ml of 0.1 M glycine-NaOH, pH 10.0, containing 0.1 M glucose and 1.0 M NaCl; (*b*) 50 ml of 0.1 M potassium phosphate, pH 7.5; and (*c*) 28 ml of 7.5 M guanidine HCl containing 0.1 M potassium phosphate, pH 7.5. Eluate 3 contained 308 μg of vitamin B_{12}-binding activity, and only 3.6% of this activity was inhibited with anti-IF antibody. This material is hereafter referred to as hog NIF.

Analysis with Vitamin B_{12}—[^{57}Co]Vitamin B_{12}, 360 μg, was added to the final preparation of hog IF, and 900 μg were added to the final preparation of hog NIF. Both preparations were dialyzed against 6.0 liters of distilled H_2O for 72 hours with dialysate changes at 24 and 48 hours. Greater than 99% of unbound vitamin B_{12} is removed under these conditions. The proteins were stored at −20°.

RESULTS

Purification of Hog IF and Hog NIF—Hog IF and hog NIF can be separated from most of the gastric proteins that do not bind vitamin B_{12} with affinity chromatography on vitamin B_{12}-Sepharose, as shown in Table I, but this technique does not result in the separation of these two proteins from each other. We have been unable to achieve satisfactory separation of hog IF from hog NIF using ammonium sulfate fractionation, ion exchange chromatography, or gel filtration on Sephadex G-150. The latter technique appeared the most promising but our preliminary studies indicated that considerable recycling would be required to achieve preparations of hog IF and hog NIF that contained less than 5% contamination with each other. We have obtained satisfactory separation of hog IF and hog NIF by the use of a method of selective affinity chromatography in which carboxylic acid derivatives of vitamin B_{12} that lack the nucleotide portion of the molecule are covalently coupled to 3,3'-diaminodipropylamine-substituted Sepharose.

Vitamin B_{12} derivatives that lack the nucleotide portion of the vitamin were prepared by hydrolysis of vitamin B_{12} as described under "Methods." Based on paper chromatograms and the observations of Armitage *et al.* (10), the lyophilized hydrolysate consisted primarily of mono- (7.9%), di- (17.3%), tri- (32.2%) and tetra- (42.6%) carboxylic acid derivatives of vitamin B_{12} that lacked the nucleotide portion of the vitamin. Hydrolysis for 5 min is sufficient to hydrolyze the nucleotide from 99% of the vitamin B_{12} molecules (10), but the hydrolysis time was increased to 1 hour to insure essentially complete nucleotide hydrolysis and to obtain a high yield of carboxyl groups which are required for coupling the vitamin B_{12} derivatives to the substituted Sepharose (8).

The mixture of carboxylic acid vitamin B_{12} derivatives was coupled to 3,3'-diaminodipropylamine-substituted Sepharose with a yield of 1.2% based on the total amount of vitamin B_{12} derivative present during the coupling reaction. We have not determined what percentage of the covalently bound vitamin B_{12} derivatives is represented by the individual mono-, di-, tri-, and tetracarboxylic acid derivatives.

It is important that selective affinity chromatography be carried out as described in Step 3 of the purification scheme (see Fig. 1) since deviations in flow rate, pH, temperature, initial guanidine concentration, and column size (*i.e.* total vitamin B_{12} derivative content) relative to sample size and content can result in decreased separation of hog IF from hog NIF. Thus, slower flow rates, neutral pH, decreased temperature, lower initial guanidine concentrations, increases in column size relative to the amount of hog IF and hog NIF applied, and substitution of vitamin B_{12}-Sepharose for the selective affinity adsorbent all have the effect of increasing the amount of hog IF that is adsorbed to the column without affecting the adsorption of hog NIF. Increases in flow rate and initial guanidine concentration as well as decreases in relative column size have the effect of decreasing the adsorption of both hog IF and hog NIF.

The first 7.5 M guanidine eluate (Eluate 4a) from the initial vitamin B_{12}-Sepharose column (see Table I) has a visible yellow color and broad but significant absorption that declines gradually from 300 to 500 nm. This colored material co-chromatographs with hog NIF during selective affinity chromatography since fraction by fraction correspondence with hog NIF could be demonstrated visually and by measurements of A_{320}. This

TABLE II

Purification of hog gastric vitamin B_{12}-binding proteins

Step	Volume	Vitamin B_{12}-binding activity		Protein	Specific activity	A_{280}:A_{361}
	ml	μg	% IF	mg	μg vitamin B_{12} bound/mg protein	
1. Hog gastric mucosal extract	875	2,620	29.6	23,600	0.111	
2. Affinity chromatography on vitamin B_{12}-Sepharose	65.0	2,130	24.4	174	12.2	
3. Selective affinity chromatography						
A. Hog IF						
A_1: fractions (11 to 13) from selective affinity chromatography	27.0	148	92.7	8.65	17.1	
A_2: repeat affinity chromatography on vitamin B_{12}-Sepharose	9.9	122	97.3	4.89	24.9	
A_3: after the addition of 360 μg vitamin B_{12} to A_2 followed by dialysis	16.3	148[a]	97.3	4.89	30.3	1.62
B. Hog NIF						
B_1: fractions (33 to 34) from selective affinity chromatography	18.0	1,010	7.6	67.4	15.0	
B_2: Sephadex G-150	22.0	366	2.1	28.9	12.7	
B_3: 3,3′-diaminodipropylamine-Sepharose	45.0	307	5.3	25.2	12.2	
B_4: repeat affinity chromatography on vitamin B_{12}-Sepharose	28.0	308	3.6	19.1	16.1	
B_5: after the addition of 900 μg vitamin B_{12} to B_4 followed by dialysis	43.7	480[a]	3.6	19.1	25.1	2.11

[a] Vitamin B_{12} content.

colored material was separated from hog NIF by gel filtration on Sephadex G-150 (see Fig. 2) where A_{320} co-chromatographed with the small A_{280} peak that was eluted prior to the major A_{280} peak that coincided with the single peak of vitamin B_{12}-binding activity. The nature of this yellow material is unknown.

A summary of the purification of hog IF and hog NIF is presented in Table II. Hog IF has been purified 920-fold with a yield of 19%. Hog NIF has been purified 320-fold with a yield of 26%. Based on protein assays performed prior to the addition of vitamin B_{12}, 1 mg of the final preparation of hog IF contains 30.3 μg of vitamin B_{12} and has an A_{280} of 1.44, an A_{361} of 0.89, and a ratio of A_{280}:A_{361} of 1.62. One milligram of the final preparation of hog NIF contains 25.1 μg of vitamin B_{12} and has an A_{280} of 1.56, an A_{361} of 0.74, and a ratio of A_{280}:A_{361} of 2.11. The final preparations of both proteins are homogeneous based on sedimentation equilibrium ultracentrifugation and polyacrylamide gel electrophoresis performed in the presence and absence of sodium dodecyl sulfate. Studies (see below) employing anti-IF antibody and pseudo-vitamin B_{12} indicate that purified hog IF and hog NIF are free of significant contamination with each other.

Removal of Vitamin B_{12}—Greater than 99% of bound vitamin B_{12} can be removed from hog IF and hog NIF by dialysis at 22° for 72 hours against 15 volumes of 0.1 M potassium phosphate, pH 7.5, containing 7.5 M guanidine HCl with dialysate changes at 24 and 48 hours. Both proteins, devoid of vitamin B_{12}, can be stored in this guanidine solution at 4° for periods up to at least 3 months without loss of vitamin B_{12}-binding activity. The ability to remove and replace vitamin B_{12} was used to increase the specific activity of [^{57}Co]vitamin B_{12} so that certain experiments such as gel filtration could be performed with small quantities of protein.

Renaturation of Hog IF and Hog NIF—The data presented in Table II (see Steps $3A_2$, $3A_3$, $3B_4$, and $3B_5$) indicate that when an excess of vitamin B_{12} was added to hog IF and hog NIF prior to dialysis to remove guanidine and unbound vitamin B_{12}, both proteins bound more vitamin B_{12} than when the two proteins were diluted 1:10,000 in guanidine-free buffer and assayed directly for vitamin B_{12}-binding activity. In other experiments, guanidine was first removed by dialysis prior to the addition of excess vitamin B_{12} and subsequent dialysis to remove unbound vitamin. In these experiments, hog IF and hog NIF both bound the same amount of vitamin B_{12} per mg of protein as was observed when excess vitamin was added prior to the removal of guanidine. This observation indicates that the presence of vitamin B_{12} is not required to achieve renaturation of these two proteins from guanidine and suggests that other factors such as protein concentration and the rate of guanidine removal may play important roles. Detailed studies concerning the renaturation process have not been conducted for hog IF and hog NIF.

TABLE III

Schilling tests performed with a patient with pernicious anemia

[^{57}Co]Vitamin B_{12} ingested	Protein ingested	Urine collected for 24 hours following the ingestion of [^{57}Co]vitamin B_{12}		
		Volume	[^{57}Co]Vitamin B_{12} content	
ng		ml	ng	% ingested amount
501	None	1490	7	1.4
501	24 μg Hog IF	1640	143	28.6
501	49 μg Hog NIF	1250	13	2.5

Schilling Tests—The results of Schilling tests performed on a single pernicious anemia patient are presented in Table III and demonstrate that 24 μg of hog IF were able to restore vitamin B_{12} absorption to a normal level. Hog NIF, at a dose of 49 μg, was ineffective.

hibition of Vitamin B_{12} Binding by Anti-IF Antibody—The ..lts of experiments performed to determine the ability of anti-IF antibody to inhibit vitamin B_{12} binding by hog IF, hog NIF, and human IF are presented in Fig. 3. At a level of 200 µl of antibody, hog IF and human IF were inhibited approximately 98%, while only approximately 3% inhibition was noted with hog NIF. The inhibition curves obtained suggest that this particular antibody has a significantly lower affinity for hog IF than for human IF.

Interaction with Pseudo-vitamin B_{12}—Samples of hog IF, hog NIF, and human IF in 7.5 M guanidine HCl were diluted 1:10,000 in 0.1 M potassium phosphate, pH 7.5, and utilized to study the ability of pseudo-vitamin B_{12} to block vitamin B_{12} binding by these proteins at 4°. The results are presented in Table IV, and the 22-hour data indicate that hog IF and hog NIF contain less than 5% contamination with each other. The fact that prior incubation with pseudo-vitamin B_{12} results in a definite decrease in the rate of [^{57}Co]vitamin B_{12} binding by hog IF indicates that this protein does bind pseudo-vitamin B_{12}, although with a lower affinity than for vitamin B_{12}. The fact that prior incubation with pseudo-vitamin B_{12} slows the rate of subsequent [^{57}Co]vitamin B_{12} binding by human IF to a lesser degree than for hog IF suggests that human IF may bind pseudo-vitamin B_{12} with a lower affinity relative to vitamin B_{12} than does hog IF. No definite conclusion can be reached in regard to this latter point, however, since association and dissociation rates will obviously influence the time course data presented in Table IV.

Polyacrylamide Disc Gel Electrophoresis—The results of the polyacrylamide disc gel electrophoresis experiments are presented in Fig. 4. Single protein bands were observed in the absence of vitamin B_{12} with 25 µg of hog IF and 25 µg of hog NIF (*Gels A* and *C*, Fig. 4). When 25 µg of hog IF saturated with vitamin B_{12} were studied, a series of protein bands was observed that appeared closer and closer together as one approached the top of the gel (*Gel B*, Fig. 4). This finding suggests that hog IF aggregates in the presence of vitamin B_{12} to

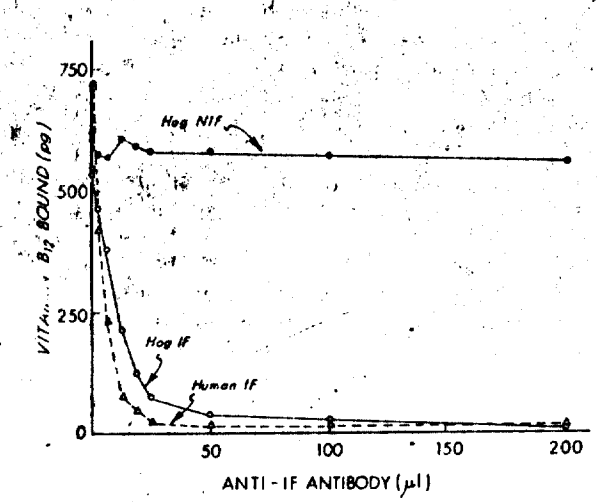

FIG. 3. Anti-IF antibody studies. Constant amounts of hog IF, hog NIF, and human IF were incubated at 22° for 30 min with varying amounts (0 to 200 µl) of anti-IF antibody obtained from the serum of a patient with pernicious anemia. At the end of a 30-min incubation period, 1000 pg of [^{57}Co]vitamin B_{12} were added; after an additional 20 min, the amount of vitamin bound was determined using the charcoal adsorption method. The amount of [^{57}Co]vitamin B_{12} bound was plotted *versus* the amount of antibody present. ●, hog NIF; ○, hog IF; △, human IF.

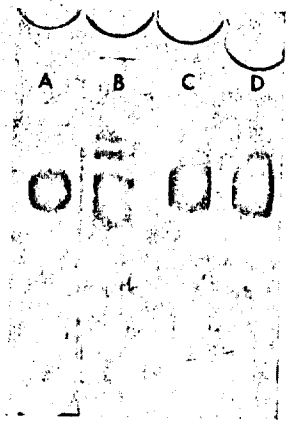

FIG. 4. Polyacrylamide disc gel electrophoresis of hog IF and hog NIF in the absence and presence of vitamin B_{12}. Protein samples devoid of vitamin B_{12} were renatured from guanidine by dialysis against H_2O for 72 hours at 4°. Protein samples saturated with vitamin B_{12} were renatured as described above except that excess vitamin B_{12} was added prior to dialysis. *Gel A*, 25 µg of hog IF devoid of vitamin B_{12}; *Gel B*, 25 µg of hog IF containing 0.76 µg of bound vitamin B_{12}; *Gel C*, 25 µg of hog NIF devoid of vitamin B_{12}; *Gel D*, 25 µg of hog NIF containing 0.63 µg of bound vitamin B_{12}. All four gels were subjected to electrophoresis at the same time. Electrophoresis was terminated when the tracking dye approached the bottom of the gels.

TABLE IV

Interaction of pseudo-vitamin B_{12} with gastric vitamin B_{12}-binding proteins

Item	Nonradioactive compound present during 30-min preincubation	[^{57}Co]Vitamin B_{12} bound at different time periods following the addition of 1000 pg							
		0.4 min	1.0 min	2.0 min	5.0 min	30 min	2 hr	22 hr	
		pg	pg	pg	pg	pg	pg	pg	%
Hog IF	None	176	323	344	401	533	548	690	100.0
Hog IF	1500 pg pseudo-vitamin B_{12}	93	107	126	134	303	456	662	95.3
Hog IF	1500 pg vitamin B_{12}	0	0	1	0	6	12	46	7.9
Hog NIF	None	255	397	516	657	892	906	972	100.0
IF	1500 pg pseudo-vitamin B_{12}	0	0	0	0	0	7	19	1.9
F	1500 pg vitamin B_{12}	0	0	0	0	0	1	10	1.0
Human IF	None	148	160	221	303	550	548	613	100.0
Human IF	1500 pg pseudo-vitamin B_{12}	95	127	168	291	477	537	594	97.0
Human IF	1500 pg vitamin B_{12}	0	1	1	1	9	16	50	8.1

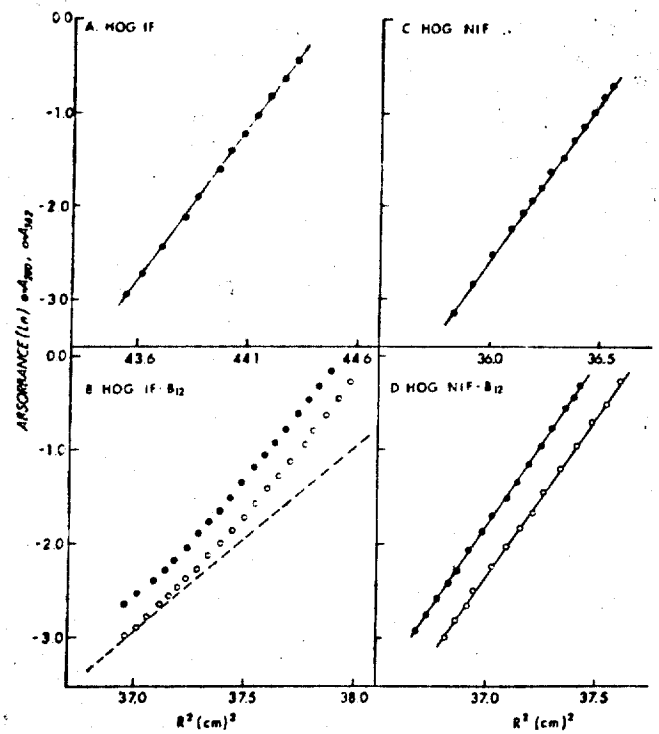

FIG. 5. Sedimentation equilibrium ultracentrifugation studies of hog IF and hog NIF in the absence and presence of vitamin B_{12}. Protein samples devoid of vitamin B_{12} were renatured from guanidine by dialysis against H_2O for 72 hours at 4°. Protein samples saturated with vitamin B_{12} were renatured as described above except that excess vitamin B_{12} was added prior to dialysis. All protein samples were dialyzed against 0.05 M potassium phosphate, pH 7.5, containing 0.75 M NaCl for 24 hours at 4° prior to sedimentation equilibrium ultracentrifugation. *A*, the sample contained 438 μg per ml of hog IF devoid of vitamin B_{12}. Centrifugation was performed for 24 hours at 5.4° at a rotor speed of 32,000 rpm. *B*, the sample contained 292 μg per ml of hog IF and 8.8 μg per ml of vitamin B_{12}. Centrifugation was performed at 6.0° for 24 hours at 24,000 rpm. *C*, the sample contained 470 μg per ml of hog NIF devoid of vitamin B_{12}. Centrifugation was performed at 5.4° for 24 hours at a rotor speed of 28,000 rpm. *D*, the sample contained 470 μg per ml of hog NIF and 11.8 μg per ml of vitamin B_{12}. Centrifugation was performed at 21° for 24 hours at a rotor speed of 30,000 rpm.

form a series of oligomers. Apparent aggregation of this type was also observed in experiments employing gel filtration and sedimentation equilibrium ultracentrifugation (see below). When 25 μg of hog NIF saturated with vitamin B_{12} were studied, the pattern (*Gel D*, Fig. 4) was the same as that observed for this protein in the absence of vitamin B_{12}, except that a faint protein band was observed above the single major protein band. It is not clear whether this faint band is due to a slight degree of aggregation by hog NIF or represents trace contamination with hog IF. Gel filtration and sedimentation equilibrium ultracentrifugation studies (see below) also indicate that hog NIF does not aggregate significantly in the presence of vitamin B_{12}.

Molecular Weight Determination by Sedimentation Equilibrium—When samples of hog IF and hog NIF were studied by sedimentation equilibrium ultracentrifugation in the absence of vitamin B_{12}, straight lines were observed in both cases when ln A_{280} was plotted *versus* R^2 (Fig. 5, *A* and *C*). Using their respective partial specific volumes of 0.714 and 0.700, calculated from the amino acid and carbohydrate analyses (see below), molecular weight values of 52,300 and 66,000 were obtained for hog IF and hog NIF.

When a sample of hog NIF saturated with vitamin B_{12} was studied under similar conditions, straight lines were obtained for plots of ln A_{280} *versus* R^2 and ln A_{362} *versus* R^2 (Fig. 5*D*). Molecular weight values of 63,100 and 61,300 were obtained for the hog NIF-vitamin B_{12} complex using the A_{280} and A_{362} data, respectively. These two values are not significantly different from each other and demonstrate correspondence between protein and vitamin B_{12}. The molecular weight values obtained for the hog NIF-vitamin B_{12} complex (63,100 and 61,300) are not significantly different from the value of 66,000 obtained for hog NIF devoid of vitamin B_{12}. This fact indicates that hog NIF did not aggregate in the presence of vitamin B_{12} under the conditions in which this experiment was performed.

When a sample of hog IF saturated with vitamin B_{12} was studied, plots of ln A_{280} *versus* R^2 and ln A_{362} *versus* R^2 revealed significant curvature (Fig. 5*B*). The fact that the degree of curvature is the same for both plots indicates correspondence between protein and vitamin B_{12}. When portions of the two curves were used for molecular weight calculations, values ranging from 60,000 to 90,000 were obtained in each case. This range of molecular weight values indicates that the hog IF-vitamin B_{12} complex existed as a mixture of monomers and higher molecular weight oligomers under the conditions in which this experiment was performed.

Amino Acid and Carbohydrate Composition—The results of the amino acid and carbohydrate analyses are presented in Table V. The values presented represent the average of two separate analyses for each protein. Hog IF contains 17.5% carbohydrate and hog NIF contains 35.5%. Based on the molecular weights of the individual amino acids and carbohydrates determined, hog IF contains 58,600 g of amino acid and carbohydrate per mole of bound vitamin B_{12}. A value of 64,000 g of amino acid and carbohydrate per mole of bound vitamin B_{12} was obtained for hog NIF. These values are close to the molecular weight values obtained for hog IF (52,300) and hog NIF (63,500) by sedimentation equilibrium ultracentrifugation (see above) and indicate that both proteins contain single vitamin B_{12}-binding sites.

The amino acid and carbohydrate compositions of hog IF and hog NIF are quite similar, but significant differences do exist. Hog IF contains significantly greater amounts of threonine, proline, and methionine than hog NIF, while the latter protein contains significantly more of each of the six carbohydrates that are present in both proteins.

The sulfhydryl group content of hog IF and hog NIF was assayed in 0.1 M potassium phosphate containing 7.5 M guanidine HCl. No free sulfhydryl groups were detected (<0.1 residue per mole), suggesting that all of the cysteine residues present in these proteins are involved in disulfide bonds.

Molecular Weight Determinations by Gel Filtration—When samples of hog IF and hog NIF devoid of vitamin B_{12} were subjected to gel filtration on Sephadex G-150, single peaks of vitamin B_{12}-binding activity were observed for both proteins (Fig. 6, *A* and *C*). Based on the elution positions of the two proteins, an apparent molecular weight of 74,000 was calculated for hog IF and a value of 130,000 was calculated for hog NIF. These values are significantly higher than the molecular weights obtained for these two proteins using sedimentation equilibrium ultracentrifugation and amino acid and carbohydrate analyses (see above). Protein aggregation appears to be an unlikely explanation for the discrepancies since gel filtration was performed at lower protein concentrations and in the same buffer as was employed for sedimentation equilibrium ultra-

TABLE V

Amino acid and carbohydrate composition of hog IF and hog NIF

Amino acid analysis was performed on duplicate 22-hour hydrolysates, and carbohydrate analysis was performed by gas-liquid chromatography.

Item	Residues per mole of bound vitamin B_{12}	
	Hog IF	Hog NIF
Amino acid		
Lysine	17	21
Histidine	9	8
Arginine	12	12
Aspartic acid	50	47
Threonine	37	19
Serine	36	29
Glutamic acid	48	42
Proline	31	15
Glycine	25	23
Alanine	32	24
Valine	26	24
Isoleucine	25	26
Leucine	43	36
Tyrosine	9	11
Phenylalanine	14	12
Methionine	10[a]	6[a]
Half-cystine	9[b]	10[b]
Tryptophan	6[c]	6[c]
Carbohydrate		
Fucose	7	25
Galactose	8	16
Mannose	15	26
Galactosamine	8 (7)[d]	16 (9)[d]
Glucosamine	12 (13)[d]	30 (25)[d]
Sialic acid	5[e]	10[e]

[a] Determined as methionine sulfone after performic acid oxidation.

[b] Determined as carboxymethylcysteine. Accurate quantitation as cysteic acid was not possible since ninhydrin-positive material was present in the cysteic acid region in the absence of performic acid oxidation.

[c] Determined spectrophotometrically.

[d] Values in parentheses were determined using the amino acid analyzer.

[e] Determined by the thiobarbiturate method.

centrifugation. A more likely explanation is that the gel filtration molecular weight values are falsely elevated due to the fact that hog IF and hog NIF contain, respectively, 17.5% and 35.5% carbohydrate. This phenomenon has been reported for other glycoproteins (9, 11, 12).

When a sample of the hog NIF-vitamin B_{12} complex was studied by gel filtration, the result presented in Fig. 6*D* was obtained. Under these conditions, hog NIF eluted as a simple symmetrical peak with an apparent molecular weight of 128,000, which is essentially the same as the value of 130,000 that was obtained when the protein was eluted in the absence of vitamin B_{12} (Fig. 6*C*). This observation indicates that hog NIF did not aggregate in the presence of vitamin B_{12} under the conditions which this experiment was performed.

When the hog IF-vitamin B_{12} complex was studied (Fig. 6*B*), two peaks of [^{57}Co]vitamin B_{12} were observed with apparent molecular weights of 75,000 and 160,000. This indicates that a mixture of hog IF monomers and dimers was present, and that aggregation in the presence of vitamin B_{12} had occurred. The fact that a shoulder is present on the leading edge of the 160,000 molecular weight peak (Fig. 6*B*) suggests that trimers or tetramers of hog IF may also have been present.

The experiments presented in Fig. 6, *B* and *D* were repeated under identical conditions except that the protein samples were incubated in 0.5 ml of gel filtration buffer at 37° for 8 hours prior to being cooled to 4° and placed on the Sephadex G-150 column. In both cases, no change in the elution pattern of [^{57}Co]vitamin B_{12} was observed.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis—When 30 μg of hog IF and hog NIF were subjected to sodium dodecyl polyacrylamide gel electrophoresis, a single protein band was observed in each case (Fig. 7). Molecular weight estimates of 66,000 and 100,000 were obtained for hog IF and hog NIF, respectively, based on their mobilities under these conditions. These values are similar to the values obtained for these proteins by gel filtration and suggest that hog IF and hog NIF both contain single polypeptide chains. The molecular weight values obtained by sodium dodecyl sulfate polyacrylamide gel electrophoresis appear to be falsely elevated as has been reported for other glycoproteins studied by this technique (9, 12–14).

Equilibrium Dialysis—The results of equilibrium dialysis experiments are presented in Fig. 8. A value of 1.5×10^{10} M^{-1} was obtained for the association constant, K_A, for hog IF and vitamin B_{12}. A value of 1.3×10^{10} M^{-1} was obtained for hog NIF and vitamin B_{12}. These two values are not significantly different and are similar to the value of 1.5×10^{10} M^{-1} obtained for human IF and vitamin B_{12} under the same conditions (9).

Absorption Spectra—The spectra of equal concentrations of the hog IF-vitamin B_{12} complex, the hog NIF-vitamin B_{12} complex, and unbound vitamin B_{12} are presented in Fig. 9. When vitamin B_{12} is bound to either of these proteins, the 361 nm spectral maximum for unbound vitamin B_{12} shifts to 362 nm.

When vitamin B_{12} binds to either hog IF or hog NIF, there is general enhancement of the vitamin B_{12} spectrum above 320 nm since the spectra of the two proteins devoid of vitamin B_{12} are those of typical proteins with insufficient absorption above 320 nm to account for the differences between the protein-bound vitamin B_{12} spectra and that of unbound vitamin B_{12} (see Fig. 9) in the region above 320 nm. This observation is supported by experiments in which subsaturating aliquots of vitamin B_{12} were added to buffer containing hog IF and hog NIF and the increase in absorption at 361 nm was noted to be approximately 30% greater than when aliquots of vitamin B_{12} were added to buffer alone.

DISCUSSION

Hog IF and hog NIF have been isolated from a crude extract of hog pyloric mucosa. Affinity chromatography on vitamin B_{12}-Sepharose served to remove most proteins that do not bind vitamin B_{12}, but extreme difficulty was encountered in attempts to separate the two vitamin B_{12}-binding proteins from each other. Holdsworth (2) and Ellenbogen *et al.* (1, 7) have succeeded in at least partially separating these two proteins by the use of ion exchange chromatography, but we and other investigators (4) have not had success with this technique. The reasons for this discrepancy are not clear, but they may involve the fact that both Holdsworth and Ellenbogen *et al.* employed incubation with proteolytic enzymes at early stages in their purification schemes since it is possible that limited proteolysis changes the chromatographic behavior of hog IF and hog NIF.

Several reports (1, 15) suggest that hog IF and hog NIF differ

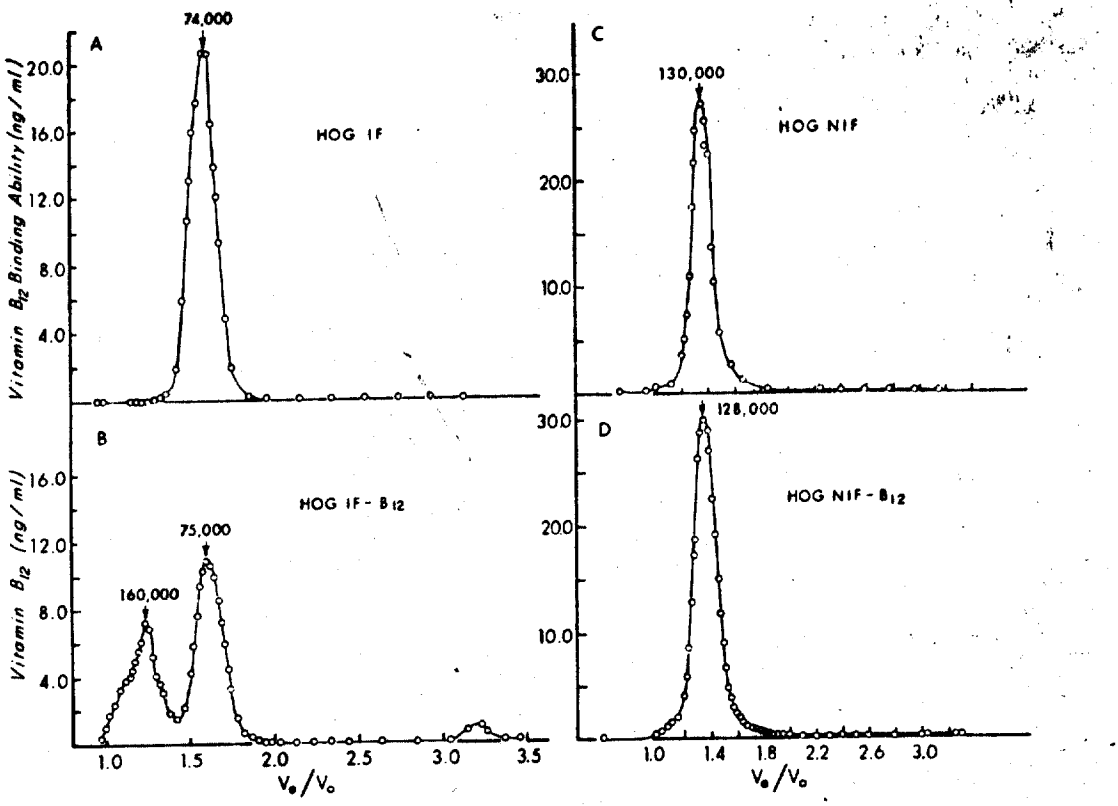

FIG. 6. Gel filtration studies of hog IF and hog NIF in the absence and presence of vitamin B_{12}. Experiments were performed at 4° using a column (2.0 × 95 cm) of Sephadex G-150 equilibrated with 0.05 M potassium phosphate, pH 7.5, containing 0.75 M NaCl. Protein samples devoid of vitamin B_{12} were renatured from guanidine by dialysis against Sephadex G-150 buffer for 72 hours at 4°. Protein samples saturated with vitamin B_{12} were renatured as described above except that excess [^{57}Co]vitamin B_{12} was added prior to dialysis. *A*, the sample contained 21 μg of hog IF devoid of vitamin B_{12}. A molecular weight of 74,000 was obtained for hog IF based on the elution position of the single peak of vitamin B_{12}-binding activity. *B*, the sample contained 21 μg of hog IF and 0.64 μg of [^{57}Co]vitamin B_{12}. Molecular weights of 75,000 and 160,000 were obtained for hog IF based on the elution position of the two peaks of [^{57}Co]vitamin B_{12}. *C*, the sample contained 36 μg of hog NIF devoid of vitamin B_{12}. A molecular weight of 130,000 was obtained for hog NIF based on the elution position of the single peak of vitamin B_{12}-binding activity. *D*, the sample contained 36 μg of hog NIF and 0.90 μg of [^{57}Co]vitamin B_{12}. A molecular weight of 128,000 was obtained for hog NIF based on the elution position of the single peak of [^{57}Co]vitamin B_{12}. The amount of vitamin B_{12}-binding activity, and [^{57}Co]vitamin B_{12}, recovered after gel filtration ranged from 72 to 92% of the amounts applied in these experiments.

FIG. 7. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of hog IF and hog NIF. *Gel A*, 30 μg of hog IF. A molecular weight of 66,000 is indicated by the mobility of the protein band. *Gel B*, 30 μg of hog NIF. A molecular weight of 100,000 is indicated by the mobility of this protein band. The *arrow* indicates the direction of mobility.

from each other in their affinities for vitamin B_{12} analogs that contain absent or structurally modified nucleotides. We have taken advantage of these observations and have synthesized an affinity adsorbant that contains covalently bound vitamin B_{12} derivatives that lack the nucleotide portion of the native vitamin. Hog IF has a marked affinity for this adsorbant but under certain conditions this affinity is less than that of hog NIF, and this fact has enabled us to separate these two proteins.

We have demonstrated that hog IF contains significantly more threonine, proline, and methionine and significantly less fucose, mannose, galactose, galactosamine, glucosamine, and sialic acid than does hog NIF. The differences between our results and those of previous investigators (see introductary section) are probably attributable to the fact that previous preparations of hog IF (1, 2, 7) appear to have contained significant contamination with other proteins. This suggestion is supported by the fact that previous preparations of hog IF had lower specific activities than ours.

Under certain conditions, hog IF appears to exist as a mixture of monomers, dimers, and other higher molecular weight oligomers. This phenomenon has been observed in studies employing polyacrylamide disc gel electrophoresis, sedimentation equilibrium ultracentrifugation, and gel filtration on Sephadex G-150. The presence of vitamin B_{12} appears to be a requirement for oligomer formation. Similar studies have failed to provide evidence for significant oligomer formation by hog NIF. This observation appears to conflict with the observations of Ellenbogen *et al.* (1, 7) since they obtained ultracentrifugational evidence that both hog IF and hog NIF were capable of forming oligomers in the presence of vitamin B_{12}. We do not have any definite explanation for this discrepancy, but it is possible that hog NIF is capable of forming oligomers only with different salt or protein concentrations than we have employed. Ellenbogen *et al.* employed incubation with trypsin and chymotrypsin as part of their purification scheme and it is also possible that limited proteolysis of hog NIF must occur before this protein is capable of oligomer formation.

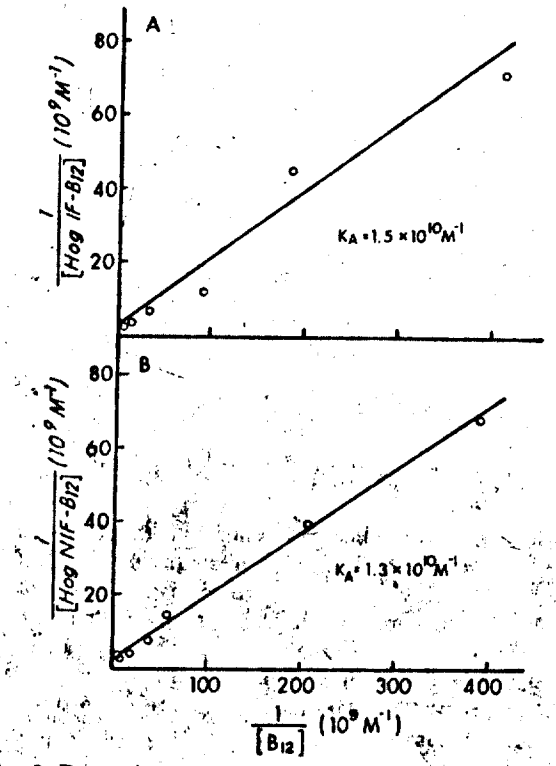

FIG. 8. Determination of the association constant, K_A, for hog IF and vitamin B_{12} (*A*) and for hog NIF and vitamin B_{12} (*B*). The experimental points were obtained using equilibrium dialysis in 0.1 M potassium phosphate, pH 7.5, at 4°.

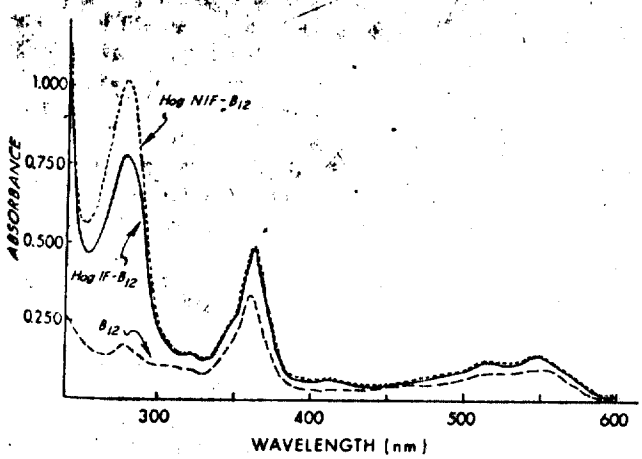

FIG. 9. Absorption spectra. ——, 540 μg per ml of hog IF containing 16.4 μg per ml of vitamin B_{12}. ····, 653 μg per ml of hog NIF containing 16.4 μg per ml of vitamin B_{12}. – – –, 16.4 μg per ml of vitamin B_{12}. All spectra were obtained at room temperature in 0.05 M potassium phosphate, pH 7.5, containing 0.75 M NaCl.

Hog IF and hog NIF have a number of properties in common but the two proteins also differ in a number of respects. Similar properties include: (*a*) both proteins have single vitamin B_{12}-binding sites; (*b*) both proteins have affinity constants for vitamin B_{12} in the range of 1.3 to 1.5 × 10^{10} M^{-1}; (*c*) both proteins contain single polypeptide chains; (*d*) neither protein has any demonstrable free sulfhydryl groups; (*e*) when vitamin B_{12} binds to either protein, there is general enhancement of the vitamin B_{12} spectrum above 320 nm; the spectra of the two protein-vitamin B_{12} complexes are indistinguishable in this region; (*f*) the 361 nm spectral maximum for unbound vitamin B_{12} shifts to 362 nm when the vitamin is bound to either protein; (*g*) both proteins have molecular weights close to 60,000, although the molecular weight of hog IF is slightly less than that of hog NIF; (*h*) hog IF and hog NIF are both glycoproteins and contain the same kind of carbohydrate residues.

Differences between hog IF and hog NIF include: (*a*) the vitamin B_{12}-binding ability of hog IF is inhibited by antibody obtained from a patient with pernicious anemia, while that of hog NIF is not. (*b*) Hog IF has a lower affinity for pseudo-vitamin B_{12} relative to vitamin B_{12} than does hog NIF. (*c*) Hog IF is able to correct vitamin B_{12} malabsorption in a patient with pernicious anemia, while hog NIF cannot. (*d*) Hog IF facilitates vitamin B_{12} binding to homogenates of human and guinea pig distal small intestine, while hog NIF does not.[2] (*e*) Hog IF and hog NIF have markedly different apparent molecular weights when determined by gel filtration. Both values are falsely elevated but the degree of false elevation is greater for hog NIF. (*f*) The two proteins also have significantly different apparent molecular weights when determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The degree of false elevation in these values is also greater for hog NIF. (*g*) Hog IF aggregates in the presence of vitamin B_{12} under conditions in which hog NIF does not. (*h*) The hog IF-vitamin B_{12} complex contains less absorption from 250 to 290 nm than the hog NIF-vitamin B_{12} complex. (*i*) Hog IF contains significantly greater amounts of threonine, proline, and methionine than hog NIF. (*j*) Hog IF contains 17.5% carbohydrate, while hog NIF contains 35.5%. Hog IF contains significantly lower amounts of fucose, galactose, mannose, galactosamine, glucosamine, and sialic acid than hog NIF.

The differences observed between hog IF and hog NIF demonstrate that these two proteins are distinct entities. The fact that hog NIF has a slightly larger molecular weight than hog IF and contains approximately twice as much carbohydrate rules out the possibility that hog NIF is a limited degradation product of hog IF. The fact that hog IF contains significantly greater amounts of threonine, proline, and methionine rules out the possibility that hog NIF is a zymogen-like precursor of hog IF. It is conceivable that both proteins are derived from a common, larger molecular weight glycoprotein, but we are not aware of any evidence to suggest such a possibility.

The function of hog NIF is unknown and our studies have not provided any clues concerning this question. Aro and Gräsbeck (4) have demonstrated that hog NIF reacts with antibodies prepared against crude hog granulocyte extracts and have suggested that hog NIF is a R type vitamin B_{12}-binding protein since members of this group of proteins have immunologic similarities and are present in granulocytes and a variety of body fluids. It is of interest in this regard that hog NIF does have many properties in common with the human granulocyte vitamin B_{12}-binding protein (12). Both proteins have similar amino acid and carbohydrate compositions and similar molecular weights; both proteins also give rise to the same degree of falsely high estimates of molecular weight when values are determined by gel filtration and sodium dodecyl sulfate polyacrylamide gel electrophoresis. The assignment of hog NIF as a member of the R type group of vitamin B_{12}-binding proteins does not, however, provide any information concerning the function of hog NIF, since specific functions have not been determined for any of the R type proteins (16).

[2] Unpublished experiments performed in collaboration with Mr. David Hooper and Dr. David Alpers of Washington University School of Medicine.

Hog IF and human IF (9) have many common properties, but the two proteins do differ in amino acid and carbohydrate composition, molecular weight, and in their interactions with anti-IF antibody and pseudo-vitamin B_{12}. The physiologic significance of these differences is unclear and they may reflect merely a species difference. It is important to note, however, that hog IF was isolated from gastric mucosa, while human IF was isolated from gastric juice. This difference could be important if IF is altered by proteolytic enzymes, or other factors, prior to, during, or after it is secreted into a gastric juice.

Acknowledgments—In addition to the people acknowledged in the accompanying paper (9), we would like to thank Dr. Leon Ellenbogen for his help in obtaining information concerning intrinsic factor concentrate.

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Isolation of Vitamin B_{12}-binding Proteins Using Affinity Chromatography

III. PURIFICATION AND PROPERTIES OF HUMAN PLASMA TRANSCOBALAMIN II*

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SUMMARY

Transcobalamin II has been isolated from Cohn Fraction III derived from 1,400 liters of pooled human plasma, using affinity chromatography on vitamin B_{12}-Sepharose and several conventional purification techniques. The final preparation was purified 2 million-fold relative to human plasma with a yield of 12.8% and was homogeneous based on polyacrylamide disc gel electrophoresis, sedimentation equilibrium ultracentrifugation, and chromatography on Sephadex G-150. Transcobalamin II binds 28.6 μg of vitamin B_{12} per mg of protein and contains one vitamin B_{12}-binding site per 59,500 g of protein as determined by amino acid analysis. The molecular weight determined by sedimentation equilibrium ultracentrifugation was 53,900 and by gel filtration on Sephadex G-150 was 60,000. Sodium dodecyl sulfate polyacrylamide gel electrophoresis disclosed two peptides with molecular weight values of 38,000 and 25,000 which suggests that transcobalamin II contains 2 subunits.

When vitamin B_{12} binds to transcobalamin II there is a shift in the peak of vitamin B_{12} absorption from 361 nm to 364 nm. Analysis of transcobalamin II for carbohydrate content using gas-liquid chromatography and amino sugar analysis by the amino acid analyzer suggest that this trace plasma protein is not a glycoprotein.

Human plasma contains two vitamin B_{12}-binding proteins in approximately equal concentration which can be distinguished from each other on the basis of a number of physical and functional parameters. The first of these proteins, transcobalamin I, has a molecular weight of approximately 120,000 as determined by gel filtration and does not bind to Cm-Sephadex at pH 6.0. The second major plasma vitamin B_{12}-binding protein, transcobalamin II, has a molecular weight of 36,000 to 38,000 by gel filtration and does bind to Cm-Sephadex at pH 6.0 (2).

Several investigators have recently postulated the existence of a transcobalamin III based on the finding of two peaks of 120,000 molecular weight vitamin B_{12}-binding protein when human plasma is fractionated on DEAE-cellulose (3, 4). It has not been established whether the heterogeneity observed for the 120,000 molecular weight vitamin B_{12}-binding protein is a result of microheterogeneity of transcobalamin I or whether it reflects the existence of separate protein species with major structural differences. Questions of this type are difficult to resolve using crude human plasma.

Transcobalamin I contains approximately 80% of the vitamin B_{12} found in normal plasma (5) and vitamin B_{12} bound to this protein has a plasma half-life of approximately 9 days (6). A specific transport function for transcobalamin I has not been defined.

Transcobalamin II is postulated to function in vitamin B_{12} transport. It has been observed *in vivo* that after physiological levels of [^{57}Co]vitamin-B_{12} are absorbed from the ileum, the vitamin appears in the plasma bound almost exclusively to transcobalamin II (7). Vitamin B_{12} bound to transcobalamin II in plasma is cleared primarily by the liver and has a plasma half-time of 12 hours (8). Studies conducted *in vitro* have demonstrated that crude preparations containing transcobalamin II facilitate the cellular uptake of vitamin B_{12} by human reticulocytes (9, 10), HeLa cells (11, 12) and Ehrlich ascites tumor cells (11). Significantly greater amounts of vitamin B_{12} are taken up from culture media by these cells when vitamin B_{12} is bound to transcobalamin II than when vitamin B_{12} is present in unbound form or is bound to other vitamin B_{12}-binding proteins such as transcobalamin I or intrinsic factor.

Additional studies concerning the plasma vitamin B_{12}-binding proteins have been limited by the fact that the vitamin B_{12}-binding capacity of human plasma is less than 2 μg per liter (3, 4). Using the molecular weight values obtained by gel filtration and assuming one vitamin B_{12}-binding site per molecule of vitamin B_{12}-binding protein, 1 liter of human plasma contains less than 100 μg of either transcobalamin I or transcobalamin II. Purification in excess of a million-fold would be required to achieve homogeneity for either of the proteins, and

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this has been beyond the limits of conventional purification techniques.

Using affinity chromatography in addition to ion exchange chromatography and gel filtration, we have succeeded in isolating transcobalamin II. This report is concerned with the purification and physical properties of this protein.

EXPERIMENTAL PROCEDURES

Materials

Cohn Fraction III was obtained from the American Red Cross National Fractionation Center. Other materials were obtained as described in the first two papers in this series (13, 14).

Methods

Vitamin B_{12}-binding assays were performed using a modification (13, 14) of the method of Gottlieb *et al.* (15). Solutions containing radioactive and nonradioactive vitamin B_{12} were assayed as described in the first paper in this series (13). The isolation of monocarboxylic acid derivatives of vitamin B_{12} and their covalent attachment to 3,3′-diaminodipropylamine-substituted Sepharose using a carbodiimide was performed as described in the first paper in this series (13). The content of covalently bound vitamin B_{12} was 0.68 μmole per ml of packed Sepharose.

Protein assays, polyacrylamide disc gel electrophoresis, sodium dodecyl sulfate polyacrylamide gel electrophoresis, sedimentation equilibrium ultracentrifugation, molecular weight determinations by gel filtration, amino acid analysis, assay of sulfhydryl group content, carbohydrate analysis, and absorption and difference spectra were all performed as described in the second paper in this series (14).

Purification of Transcobalamin II

Step 1: Cohn Fraction III of Human Plasma—Transcobalamin II was purified starting with Cohn Fraction III of human plasma. All procedures were performed at 4°. Each lot of Cohn Fraction III (72 kg) was derived from approximately 3000 liters of pooled human plasma. A typical purification using 34 kg of this material is described below. The frozen material was chopped with an ice pick into pieces weighing less than 500 g and 8.5 kg of these frozen pieces of Cohn Fraction III were placed in each of four new 100-liter plastic trash containers which contained the following: 90 liters of H_2O at 4°, 124.2 g of $NaH_2PO_4 \cdot H_2O$, 29.8 g of $Na_2HPO_4 \cdot 7\ H_2O$, and 526 g of NaCl. After the addition of Cohn Fraction III each container was stirred continuously for 4 hours using a motor-driven propellor type mixer. At the end of this time the pH of the Cohn Fraction III suspension was approximately 5.8.

Step 2: Cm-Sephadex—Sixty-three grams of dry, unprocessed Cm-Sephadex-C50 were added to each container and stirring was continued for an additional 4 hours. After the addition of the Cm-Sephadex, the pH of the suspension rose to 5.9. Stirring was stopped and the Cm-Sephadex was allowed to settle overnight. The upper 85 liters of each container were next removed through a siphon and discarded. The Cm-Sephadex was collected from the 8 liters of suspension remaining in each container by suction filtration using a Buchner funnel and 24-cm diameter circles of S & S filter paper No. 585. Approximately 2 kg of Cm-Sephadex, wet weight, were recovered from each of the large plastic containers, and the Cm-Sephadex from each container was suspended in 4 liters of the original Cohn Fraction III suspension solution. After stirring for 5 min each suspension of Cm-Sephadex was again collected by suction filtration. Each batch of Cm-Sephadex was then suspended in 2 liters of 0.1 M sodium phosphate, pH 5.8, containing 1.0 M sodium chloride and stirred with a magnetic stirrer for 30 min. Each suspension was suction-filtrated on a Buchner funnel containing a 24.5-cm diameter circle of S & S glass wool No. 24 on top of a 24-cm diameter circle of S & S filter paper No. 585. The filter cake was then washed directly on the Buchner funnel with an additional 2 liters of the same eluting solution. A combined total of 19,700 ml of this elution filtrate was obtained and contained 78% of the vitamin B_{12}-binding activity present in the initial Cohn Fraction III suspension. The elution filtrate contained only a faint turbidity and was used directly for affinity chromatography on vitamin B_{12}-Sepharose.

Step 3: Affinity Chromatography on Vitamin B_{12}-Sepharose—A column 2.5 cm in diameter and 2 cm tall of vitamin B_{12}-Sepharose was prepared and washed with 100 ml of 0.1 M glycine-NaOH, pH 10.0, followed by 100 ml of 0.1 M sodium phosphate, pH 5.8, containing 1.0 M NaCl. This procedure served to remove traces of vitamin B_{12} which had become hydrolyzed from covalent linkage to Sepharose. The entire 1.0 M NaCl elution filtrate from the previous Cm-Sephadex batch step was then applied to the column of vitamin B_{12}-Sepharose with a gravity head of approximately 250 cm of water. The flow rate was approximately 500 ml per hour. Only 7.7% of the vitamin B_{12}-binding activity applied to the vitamin B_{12}-Sepharose column was recovered in the total effluent. Small aliquots of the effluent were collected directly from the vitamin B_{12}-Sepharose column at various times during the sample application. These aliquots were also assayed for vitamin B_{12}-binding activity. They indicated that early in the sample application greater than 99% of the vitamin B_{12}-binding activity was adsorbed to the vitamin B_{12}-Sepharose and that this level of adsorption had fallen to 90% near the end of the sample application. After the entire sample had been applied, the column was then washed with different volumes of a variety of solutions in the following order. Wash 1: 100 ml of 0.1 M sodium phosphate, pH 5.8, containing 1.0 M NaCl. Wash 2: 500 ml of 0.1 M potassium phosphate, pH 7.5. Wash 3: 1950 ml of 0.1 M glycine-NaOH, pH 10.0, containing 1.0 M NaCl and 0.1 M glucose. Wash 4: 300 ml of 0.1 M sodium phosphate, pH 5.8, containing 1.0 M NaCl. Wash 5: 150 ml of H_2O. Wash 6: 275 ml of 0.1 M potassium phosphate, pH 7.5. Wash 7: 100 ml of 0.1 M potassium phosphate, pH 7.5, containing 0.75 M guanidine HCl. The flow rate during the first six column washes was 200 ml per hour and that of Wash 7 was 100 ml per hour. The effluent from each wash was collected separately. At the completion of Wash 7, the flow rate was decreased to 20 ml per hour and a solution of 0.1 M potassium phosphate, pH 7.5, containing 7.5 M guanidine HCl was applied. The first 43.0 ml of column effluent were collected in their entirety and were designated as column Wash 8a. The next 5.3-ml effluent from Wash 8 was collected separately and designated as column Wash 8b. At this point the column was clamped and allowed to stand for 18 hours. At the end of this time the column was unclamped and the first 13.0 ml of effluent were collected and were designated as column Wash 8c. Each of the column effluents mentioned above was assayed for vitamin B_{12}-binding activity and, except for those fractions containing guanidine, was also assayed for protein content. The results are presented in Table I. Eluate 8a from vitamin B_{12}-Sepharose affinity chromatography was mixed with [^{57}Co]vitamin-B_{12} (1320 μg, 0.0034 μCi per μg) in a final volume of 44 ml. This mixture was dialyzed against 6 liters of 0.1 M Tris-HCl, pH 8.9, contain-

TABLE I

Affinity chromatography of transcobalamin II

Item	Volume	Vitamin B_{12}-binding activity		Protein		Flow rate
	ml	*ng/ml*	*total ng*	*mg/ml*	*total mg*	*ml/hr*
CM-Sephadex eluate applied to vitamin B_{12}-Sepharose	19,700	22.8	447,000	38.5	760,000	500
Initial effluent from vitamin B_{12}-Sepharose	19,700	1.70	33,500	39.5	778,000	
Further elutions of vitamin B_{12}-Sepharose:						
1. 0.1 M sodium phosphate, pH 5.8, 1.0 M NaCl	100	0.86	86	0.57	57	200
2. 0.1 M potassium phosphate, pH 7.5	500	0.74	370	0.18	90	200
3. 0.1 M glycine-NaOH, pH 10.0, 1.0 M NaCl, 0.1 M glucose	1,950	2.12	4,130	0.38	1,570	200
4. 0.1 M sodium phosphate, pH 7.5, 1.0 M NaCl	300	0.62	186	0.01	3	200
5. H_2O	150	0.38	57	0.00	0	200
6. 0.1 M potassium phosphate, pH 7.5	275	0.03	8	0.00	0	200
7. 0.1 M potassium phosphate, pH 7.5, 0.75 M guanidine HCl	100	0.44	44			100
8. 0.1 M potassium phosphate, pH 7.5, 7.5 M guanidine HCl:						
a. Initial eluate	43.0	4,600	198,000			20
b. Eluted immediately after 8a	5.3	30	159			20
c. Eluted 18 hours after 8b	13.0	25	325			20
Eluate 8a after addition of 1,320 μg of vitamin B_{12} followed by dialysis	62.0	5,690[a]	353,000[a]	0.398	24.7	

[a] Vitamin B_{12} content.

ing 0.2 M NaCl. After 4 hours the dialysate was changed and after an additional 24 hours the dialysate was changed to 6 liters of 0.1 M Tris-HCl, pH 8.9, without NaCl and dialysis was continued for an additional 45 hours. Greater than 99% of unbound vitamin B_{12} was removed by dialysis under these conditions. The transcobalamin II-vitamin B_{12} fraction was centrifuged at 50,000 × *g* for 30 min to remove denatured protein prior to chromatography on DEAE-cellulose.

Step 4: Chromatography on DEAE-Cellulose—A column (0.9 × 12 cm) of DEAE (Whatman DE 52) equilibrated with 0.1 M Tris-HCl, pH 8.9, was first washed with 60 ml of 0.1 M Tris-HCl, pH 8.9, containing 0.0111 μg of [^{57}Co]vitamin-B_{12} per ml (0.0034 μCi per μg) before the transcobalamin II-vitamin B_{12} fraction from Step 3 was applied to the column at a flow rate of 25 ml per hour. The column was washed with 10 ml of the [^{57}Co]vitamin-B_{12} containing equilibrating solution and then eluted with a linear gradient in which the mixing chamber contained 225 ml of 0.1 M Tris-HCl, pH 8.9, and the reservoir contained 225 ml of 0.1 M Tris-HCl, pH 8.9, containing 0.5 M NaCl. All of these eluting solutions contained [^{57}Co]vitamin-B_{12} as described above. Fractions were assayed for A_{280}, vitamin B_{12} content and conductivity. The results are presented in Fig. 1. Fractions 50 through 65 were pooled.

Step 5: Chromatography on 3,3′-Diaminodipropylamine-substituted Sepharose—A column (0.9 × 6 cm) of 3,3′-diaminodipropylamine-substituted Sepharose was equilibrated with 100 ml of 0.1 M Tris-HCl, pH 8.9, containing 0.2 M NaCl. The DEAE-cellulose pooled fractions 50 to 65 of transcobalamin II-vitamin B_{12} were applied to the column at a flow rate of 50 ml per hour and the column was eluted with this same buffer. The first 82 ml of effluent from the column contained greater than 99% of the transcobalamin II-vitamin B_{12} applied. The transcobalamin II-vitamin B_{12} solution was adjusted to contain 0.75 M NaCl and was then concentrated to approximately 1 ml using an Amicon ultrafiltrator equipped with a Diaflo UM-10 membrane. Despite stirring during the concentration procedure, a red film was observed on the Diaflo membrane at the completion of the concentrating procedure. The Amicon concentrate was removed and the concentrating vessel was rinsed repeatedly with 0.05 M potassium phosphate, pH 7.5, containing 0.75 M NaCl until the red film on the membrane went into solution. The final concentrate was slightly turbid and this precipitate was removed by centrifugation at 10,000 × *g* for 10 min. Approximately 4% of the total vitamin B_{12} present was present in the small pink precipitate, with the remaining 96% being present in the 6.0 ml of red supernatant solution. This supernatant solution was immediately subjected to chromatography on Sephadex G-150.

Step 6: Chromatography on Sephadex G-150—A column (2.0 × 90 cm) of Sephadex G-150, fine grade, was equilibrated with 0.05 M potassium phosphate, pH 7.5, containing 0.75 M NaCl and [^{57}Co]vitamin-B_{12} (0.0111 μg per ml, 0.0034 μCi per μg). The transcobalamin II-vitamin B_{12} fraction from the preceding step was applied directly to the top of the column and the column was eluted with the equilibrating solution at a flow rate of 20 ml per hour. Fractions of 3.3 ml were collected and assayed for vitamin B_{12} content and for absorption at 280 nm (Fig. 2). Fractions 51 to 64 were pooled and concentrated using an Amicon ultrafiltrator as described in Step 5. A red film also observed on the Diaflo membrane at the end of this concentration procedure was dissolved as described in Step 5. Less than 1% of the vitamin B_{12} placed in the Amicon ultrafiltrator passed through the UM-10 membrane. The Amicon concentrate and the rinses were combined, centrifuged at 10,000 × *g* for 10 min, and the red supernatant decanted. A small dark red pellet containing 2% of the total vitamin B_{12} present was discarded. The red supernatant, containing 98% of the vitamin B_{12}, was divided into 1.5-ml aliquots, quick-frozen in a Dry Ice-acetone bath, and stored at −70°. A summary of the purification procedure is presented in Table II.

RESULTS

Original attempts to purify transcobalamin II by passing plasma directly over vitamin B_{12}-Sepharose columns were unsuccessful because of the viscous nature of plasma and the fact

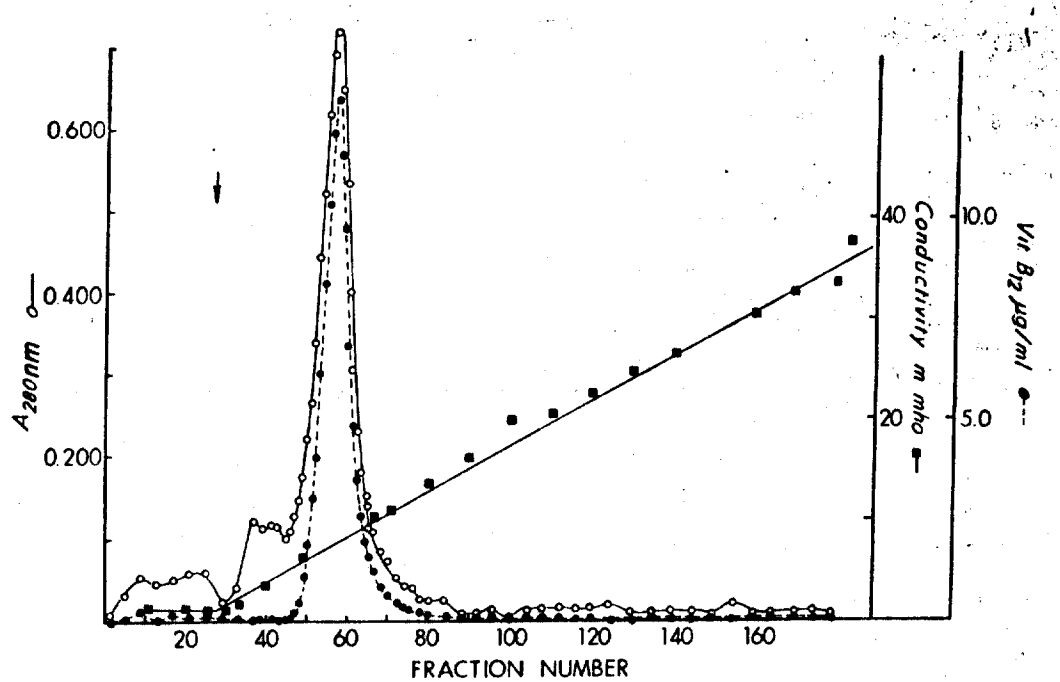

FIG. 1. Elution pattern (purification step 4) of the transcobalamin II-vitamin B_{12} complex after chromatography on a column (0.9 × 12 cm) of DEAE-cellulose. The column was equilibrated with 0.1 M Tris-HCl, pH 8.9, prior to sample application and was eluted with a linear gradient in which the mixing chamber contained 225 ml of 0.1 M Tris-HCl, pH 8.9, and the reservoir contained 225 ml of Tris-HCl, pH 8.9, 0.5 M NaCl. ○, A_{280}; ●, vitamin B_{12}; ■, conductivity. The *arrow* indicates the point at which the gradient was begun.

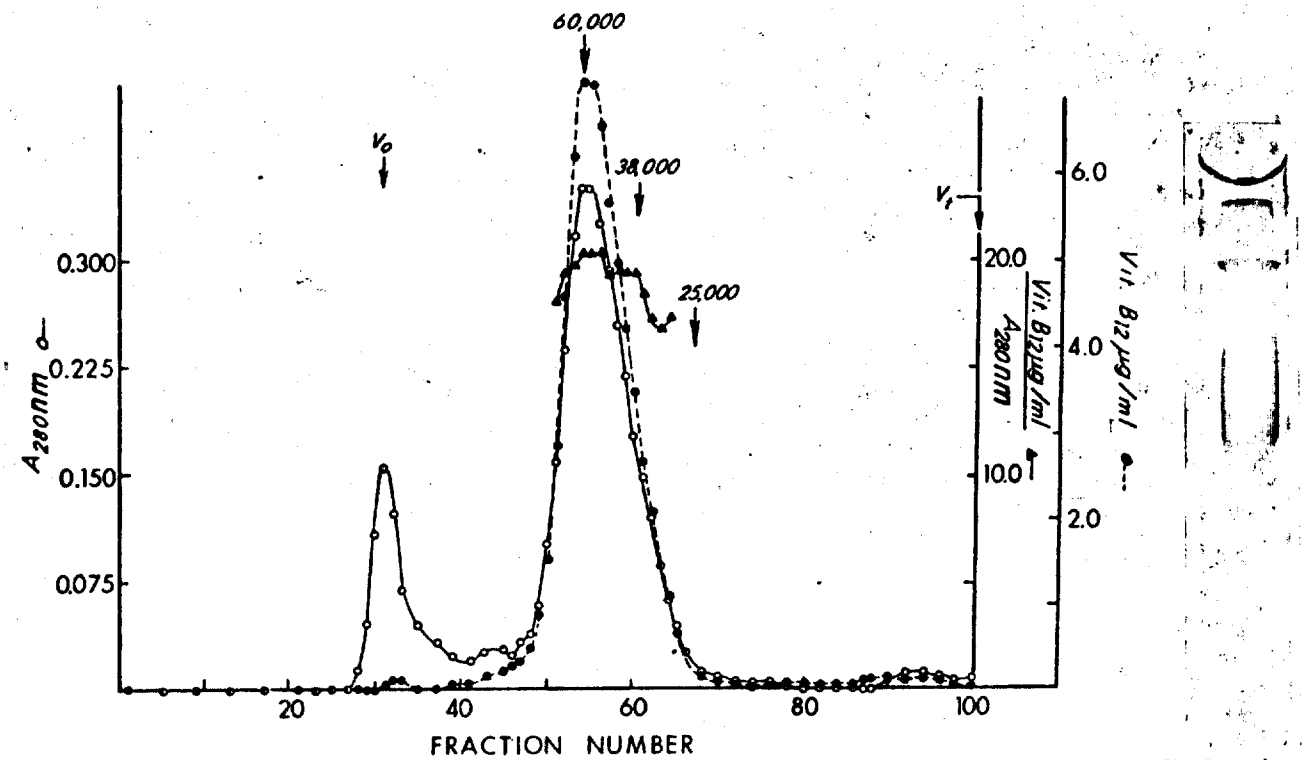

FIG. 2 (*left*). Elution profile (purification step 6) of the transcobalamin II-vitamin B_{12} complex on a column (2.0 × 90 cm) of Sephadex G-150 equilibrated with 0.05 M potassium phosphate, pH 7.5, containing 0.75 M NaCl and 0.0111 µg of [^{57}Co]vitamin-B_{12} (0.0034 µCi per µg) per ml. ○, A_{280}; ●, vitamin B_{12}; ▲, specific activity. V_0 and V_t were determined with blue dextran 2000 and dinitrophenylalanine, respectively, during eight other gel filtration experiments under the same conditions as above with less than 1% variation in their position. On the elution profile shown here the transcobalamin II-vitamin B_{12} complex has an apparent molecular weight of 60,000 as indicated (see Fig. 6). The expected elution positions for 38,000 and 25,000 molecular weight material are also indicated.

FIG. 3 (*right*). Polyacrylamide disc gel electrophoresis of 30 µg of transcobalamin II containing 1.05 µg of bound vitamin B_{12}

TABLE II

Purification of transcobalamin II

Step	Volume	Transcobalamin II-vitamin B_{12}-binding activity	Protein	Specific activity	A_{280}/A_{361}	Fold purified	Yield
	ml	*total ng*	*total mg*	*ng vitamin B_{12} bound/mg protein*			%
Human plasma	1,400,000	140,000,000[a]	98,000,000	0.0143		1	100
Cohn Fraction III	372,000	573,000	5,280,000	0.109		7.6	40.9
Cm-Sephadex eluate	19,700	447,000	760,000	0.588		41	31.9
Affinity chromatography on vitamin B_{12}-Sepharose	62.0	353,000[b]	24.7	14,000	3.56	1,000,000	25.2
DEAE-cellulose	61.5	276,000[b]	11.2	24,600	2.46	1,720,000	19.7
1,3-Diaminodipropylamine Sepharose	82.0	258,000[b]	10.2	25,400	2.38	1,780,000	18.4
Sephadex G-150	9.1	179,000[b]	6.26	28,600	2.04	2,000,000	12.8

[a] Values for protein content and transcobalamin II-vitamin B_{12}-binding activity were not determined on the plasma used in this purification. The values given are averages obtained from analysis of other human plasma samples.
[b] Based on vitamin B_{12} content.

that precipitates form during chromatography causing slow column flow rates.

Cohn Fraction III, supplied as a frozen wet paste in 72-kg lots derived from 3000 liters of pooled human plasma, was used as the starting material. We have tested five separate lots of Cohn Fraction III and have observed vitamin B_{12}-binding activities ranging from 12 to 18 ng of vitamin B_{12} bound per g of frozen, wet paste. No loss of activity has been noted after storage of Cohn Fraction III at −20° for several months. Based on the binding of the vitamin B_{12}-binding protein in Cohn Fraction III to Cm-Sephadex as well as its elution profile on Sephadex G-150 we conclude that greater than 95% of the vitamin B_{12}-binding activity in Cohn Fraction III is attributable to transcobalamin II (2). Assuming that pooled human plasma contains 1 ng per ml of transcobalamin II-vitamin B_{12}-binding activity, 27% to 40% of the transcobalamin II present in plasma is recovered in Cohn Fraction III.

As shown in Table II transcobalamin II is partially purified by batch chromatography on Cm-Sephadex before it is further purified by affinity chromatography on vitamin B_{12}-Sepharose. The Cm-Sephadex step results in a 20-fold reduction in volume and a 5-fold purification, but its major advantage is that transcobalamin II is obtained in a solution that is capable of passing over a column of vitamin B_{12}-Sepharose without clogging the column. Initial attempts to suspend Cohn Fraction III in various buffers followed by centrifugation failed to produce solutions that were suitable for direct application to vitamin B_{12}-Sepharose because of protein precipitation.

The composition of the solution used to suspend Cohn Fraction III (0.02 M sodium phosphate, pH 5.7, 0.1 M NaCl) is important since at lower concentrations of NaCl transcobalamin II does not go into solution while at higher concentrations it does not bind to Cm-Sephadex. Transcobalamin II was eluted from Cm-Sephadex with 0.1 M sodium phosphate, pH 5.8, containing 1.0 M NaCl. Transcobalamin II can also be eluted from Cm-Sephadex with 0.1 M sodium phosphate at pH values greater than 8.0, but this eluate precipitates within hours of the elution process which makes affinity chromatography on vitamin B_{12}-Sepharose impossible. Transcobalamin II is relatively unstable after elution from Cm-Sephadex since about 10% of the vitamin B_{12}-binding activity is lost per 24 hours at 4°.

No attempt has been made to determine the amount of transcobalamin II already containing bound vitamin B_{12} that is present in Cohn Fraction III, nor have we analyzed the fate of this complex during the early purification steps. Based on our finding (see below) that transcobalamin II has one vitamin B_{12}-binding site per molecule, we would not expect that transcobalamin II already containing vitamin B_{12} would be adsorbed by the vitamin B_{12}-Sepharose column.

Affinity chromatography on vitamin B_{12}-Sepharose results in a 24,000-fold purification of transcobalamin II, but approximately 50% contaminating protein is still present after this purification step. This result is in contrast to the purification of the granulocyte vitamin B_{12}-binding protein (14) where no detectable contaminating protein is present after affinity chromatography. The most likely reason for this difference resides in the fact that 98% of the granulocyte vitamin B_{12}-binding protein remained adsorbed to vitamin B_{12}-Sepharose when the column was washed with 5.0 M guanidine HCl while significant amounts of transcobalamin II are eluted with 5.0 M guanidine HCl and this washing procedure could not be employed for transcobalamin II. The comparative ease of elution of transcobalamin II is also demonstrated by the fact that only several hours of incubation with 7.5 M guanidine are required for elution (see Table I) while 41 hours are required for the granulocyte vitamin B_{12}-binding protein (14).

Transcobalamin II has been purified 2 million-fold relative to plasma with a recovery of 12.8% (Table II). The final preparation is homogeneous based on results of disc gel electrophoresis, sedimentation equilibrium ultracentrifugation, gel filtration on Sephadex G-150, and the ratio of total amino acid content to bound vitamin B_{12}. Based on the pooled Sephadex G-150 fractions, 1 mg of protein contains 28.6 μg of bound vitamin and has an A_{280} of 1.5 and an A_{361} of 0.74. The ratio of $A_{280}:A_{361}$ is 2.04.

Solubility of Transcobalamin II-Vitamin B_{12} Complex—Transcobalamin II saturated with vitamin B_{12} precipitates under a variety of conditions, *e.g.* dialysis of transcobalamin II-vitamin B_{12} (0.1 mg of protein per ml) against H_2O or 0.1 M sodium acetate pH 5.5. Detailed studies concerning transcobalamin II solubility have not been conducted but the precipitation of the transcobalamin II-vitamin B_{12} complex appears favored by high protein concentration, low pH values, and decreased ionic strength. The transcobalamin II-vitamin B_{12} complex is soluble in 0.05 M potassium phosphate containing 0.75 M NaCl at protein concentrations as high as 1 mg per ml. Solutions of this

composition were utilized for storage of the protein as well as for performing many of the physical studies outlined below.

Removal of Vitamin B_{12} from Transcobalamin II—Vitamin B_{12} can be removed from transcobalamin II by dialysis at room temperature against 7.5 M guanidine HCl containing 0.1 M potassium phosphate, pH 7.5. When transcobalamin II containing vitamin B_{12} is dialyzed against 15 volumes of this solution with changes at 24 and 48 hours, greater than 99% of the original bound vitamin B_{12} is removed in 72 hours. Transcobalamin II devoid of vitamin B_{12} can be stored in this guanidine solution at 4° for at least 3 months without significant loss of vitamin B_{12}-binding activity as assayed by adding a 3-fold excess of vitamin B_{12} (containing radioactive vitamin B_{12}) to the transcobalamin II guanidine solution, followed by removal of guanidine and unbound vitamin B_{12} by dialysis against 0.1 M potassium phosphate, pH 7.5, containing 0.75 M NaCl. The ability to remove and then replace the original bound vitamin B_{12} was used to increase the content of [^{57}Co]vitamin-B_{12} so that certain studies, such as gel filtration, could be performed with small quantities of protein.

Factors Influencing Renaturation of Transcobalamin II—The renaturation (*i.e.* vitamin B_{12}-binding ability) of transcobalamin II is greater when guanidine is removed by dialysis in the presence of a 3-fold excess of vitamin B_{12} than when aliquots of the protein in guanidine are diluted 1:5,000 and assayed for vitamin B_{12}-binding activity directly. This is illustrated in Table I where the initial 7.5 M guanidine HCl eluate from vitamin B_{12}-Sepharose bound 353,000 ng of vitamin B_{12} by the former method and only 198,000 ng by the latter method. Similar results are obtained using the final preparation of transcobalamin II. Thus, when a 3-fold excess of vitamin B_{12} is added prior to dialysis, transcobalamin II binds 27–30 μg vitamin B_{12} per mg of protein compared to a value of 14 to 18 μg of vitamin B_{12} per mg of protein when the vitamin is added after dialysis or after a 5,000 to 75,000 dilution of a solution containing the protein and guanidine. These results indicate that the presence of the vitamin is an important factor in the renaturation process. A similarly increased yield of native protein after renaturation in the presence of vitamin B_{12} was also observed for the granulocyte vitamin B_{12}-binding protein (14).

In other studies variation in protein concentration, temperature, pH, and salt concentrations as well as the addition of EDTA, sulfhydryl compounds, and glycerol have not resulted in any significant increase in the renaturation (*i.e.* vitamin B_{12}-binding activity) of transcobalamin II when guanidine is removed in the absence of vitamin B_{12}. The presence of 0.02 M 2-mercaptoethanol and dithiothreitol both cause a marked decrease in the degree of transcobalamin II renaturation.

Polyacrylamide Disc Gel Electrophoresis—When 30 μg of the transcobalamin II-vitamin B_{12} complex were subjected to polyacrylamide disc gel electrophoresis and stained for protein the pattern presented in Fig. 3 was obtained. Unstained gels had a faint red color that was localized to the entire region of the gel that stained for protein. Unstained gels were cut into 1-mm sections and the distribution of vitamin B_{12} was determined by measuring the radioactivity of the individual gel slices. A single broad peak of radioactivity was observed that coincided with the gel region that stained for protein. The reason for the failure to obtain a sharper band of either protein or vitamin B_{12} has not been determined but may be related to the limited solubility (see above) of the transcobalamin II-vitamin B_{12} complex since high protein concentrations are achieved during the stacking period of disc gel electrophoresis.

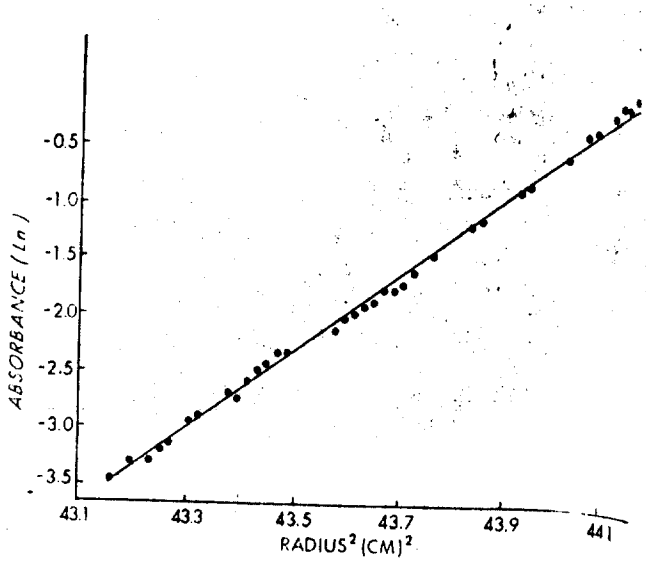

FIG. 4. Plot of ln absorbance *versus* R^2 for the transcobalamin II-vitamin B_{12} complex in 0.05 M potassium phosphate, pH 7.5, containing 0.75 M NaCl. In this experiment the protein concentration was 0.136 mg per ml and the cell was scanned at 280 nm.

Molecular Weight Determination by Sedimentation Equilibrium—Sedimentation equilibrium experiments were performed with the transcobalamin II-vitamin B_{12} complex at protein concentrations of 0.068, 0.136, and 0.204 mg per ml in 0.05 M potassium phosphate, pH 7.5, containing 0.75 M NaCl. Plots of ln A_{280} *versus* R^2 and ln A_{360} *versus* R^2 gave straight lines in all three experiments. The plot of ln A_{280} *versus* R^2 obtained at 0.136 mg of protein per ml is shown in Fig. 4. The values for the slopes of the straight lines obtained by plotting ln absorbance *versus* R^2 were the same when cells were scanned at 280 nm and 360 nm indicating correspondence between protein and vitamin B_{12}. No dependence on protein concentration was observed. Using the partial specific volume of 0.747 calculated from the amino acid analysis (see below) a molecular weight of 53,900 ± 2,360 S.D. was obtained for the transcobalamin II-vitamin B_{12} complex using the data from the 280 nm scans. When data from one of the 360 nm scans were used to calculate the molecular weight, a value of 52,800 was obtained.

Amino Acid Analysis of Transcobalamin II—The amino acid composition of transcobalamin II is presented in Table III.

When sulfhydryl groups were assayed in 7.5 M guanidine HCl containing 0.1 M potassium phosphate, pH 7.5, no sulfhydryl residues were detected (<0.1 residue per mole of transcobalamin II). This finding indicates that any cysteine residues in transcobalamin II are involved in disulfide bands.

Based on the molecular weights of the individual amino acids determined, transcobalamin II contains 59,500 g of amino acid per mole of bound vitamin B_{12}. This value is close to the respective molecular weights of 53,900 and 60,000 determined for the transcobalamin II-vitamin B_{12} complex by sedimentation equilibrium ultracentrifugation (see above) and gel filtration on Sephadex G-150 (see below). The agreement among these studies indicates that transcobalamin II contains a single vitamin B_{12}-binding site and that the final preparation of transcobalamin II is devoid of major contamination by denatured transcobalamin II or other proteins.

Carbohydrate Analysis—No carbohydrate residues were detected in the final preparation of transcobalamin II by gas-liquid chromatography and no amino sugar residues were detected on the amino acid analyzer. The amount of protein

TABLE III

Amino acid composition of transcobalamin II

Amino acid	Residues of amino acid per mole of bound vitamin B_{12}	Amino acid	Residues of amino acid per mole of bound vitamin B_{12}
Lysine	24	Alanine	42
Histidine	20	Valine	23
Arginine	25	Isoleucine	16
Aspartic acid	37	Leucine	92
Threonine	27	Tyrosine	14
Serine	37	Phenylalanine	14
Glutamic acid	71	Methionine	10[a]
Proline	27	Half-cystine	—[b]
Glycine	43	Tryptophan	6[c]

[a] Determined as methionine sulfone after performic acid oxidation.

[b] Accurate quantitation was not possible since ninhydrin-positive material was present in the cysteic acid position in the absence of performic acid oxidation. If one assumes that all of the material in this region is cysteic acid, then 9 residues were present in the standard analysis and 13 residues were present after performic acid oxidation.

[c] Determined spectrophotometrically.

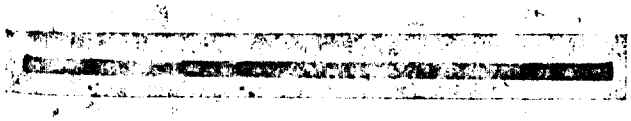

FIG. 5. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of 30 μg of transcobalamin II. Electrophoresis was performed for 7½ hours, and at the end of this time the tracking dye was located 3 cm from the bottom (*left*) of the 20-cm gel. The mobility of the two protein bands indicates molecular weights of 38,000 and 25,000.

analyzed was such that 1 mole of fucose, galactose, glucose, mannose, *N*-acetylgalactosamine, *N*-acetylglucosamine, or sialic acid per mole of bound vitamin B_{12} would have been detected. Thus, transcobalamin II is not a glycoprotein.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis—When 30 μg of the transcobalamin II-vitamin B_{12} complex were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis, two protein bands were observed that stained with Coomassie brilliant blue with equal intensity (Fig. 5). The molecular weights estimated for these polypeptides were 38,000 and 25,000.

The sum of the molecular weights of these two components is 63,000 which is similar to the molecular weight estimates for transcobalamin II, obtained by sedimentation equilibrium, amino acid analysis, and gel filtration, and suggests that transcobalamin II consists of one 38,000 molecular weight subunit and one 25,000 molecular weight subunit.

Molecular Weight Determination by Gel Filtration—Gel filtration was the final step used in the purification of transcobalamin II. This was performed on a column (2.0 × 90 cm) of Sephadex G-150 equilibrated with the same solution used for the sedimentation equilibrium studies, *i.e.* 0.05 M potassium phosphate, pH 7.5, 0.75 M NaCl. Transcobalamin II was eluted as an isolated peak with correspondence between the amount of A_{280} and vitamin B_{12} as shown in Fig. 2. Based on the empirically determined relationship between K_{av} and log molecular weight (see Fig. 6), the transcobalamin II-vitamin B_{12} complex had a molecular weight of 60,000. Sodium dodecyl sulfate gel electrophoresis has suggested that transcobalamin II consists of 2

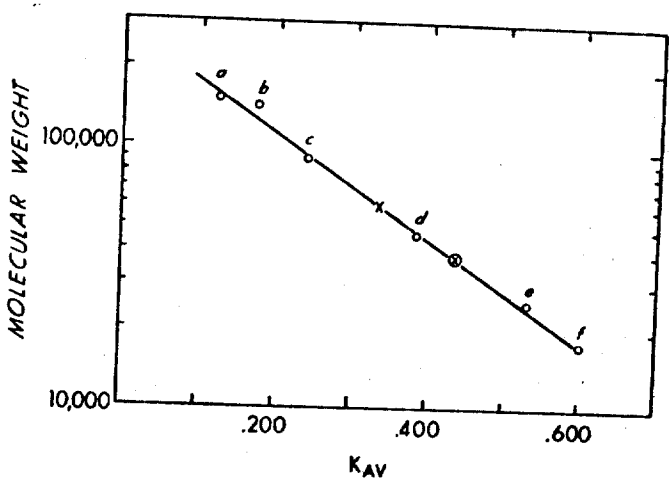

FIG. 6. Determination of the apparent molecular weight of transcobalamin II by gel filtration on a column (2.0 cm × 90 cm) of Sephadex G-150 equilibrated with 0.05 M potassium phosphate, pH 7.5, containing 0.75 M NaCl. The proteins used to calibrate the column were: *a*, IgG γ-globulin; *b*, lactic dehydrogenase; *c*, transferrin; *d*, ovalbumin; *e*, chymotrypsinogen; *f*, myoglobin. × indicates the value for K_{av} obtained for the transcobalamin II-vitamin B_{12} complex during the final purification (see Fig. 2). The apparent molecular weight obtained for the transcobalamin II-vitamin B_{12} complex from this experiment was 60,000. ⊗ indicates the value of K_{av} obtained when 40 μg of the final preparation of transcobalamin II were applied to the same column of Sephadex G-150 in the presence and absence of [^{57}Co]vitamin-B_{12}. An apparent molecular weight of 38,000 was obtained in both of these experiments. (See text for additional details and comment.)

subunits of 38,000 and 25,000 molecular weight, and it is important to note that during the final purification on Sephadex G-150 no shoulder of A_{280} or vitamin B_{12} content was observed at the 38,000 molecular weight position and almost no A_{280} or vitamin B_{12} was present at the 25,000 molecular weight position. This observation suggests that the 2 transcobalamin II subunits were associated during the Sephadex G-150 final purification step.

Other gel filtration experiments were performed on the same Sephadex G-150 column using samples of transcobalamin II which were 250-fold less concentrated with respect to protein than in the experiment described above. Eighty micrograms of the isolated protein were dialyzed against 7.5 M guanidine HCl to remove greater than 99% of the bound vitamin B_{12}. Half of this protein solution was then dialyzed against 300 volumes of 0.05 M potassium phosphate, pH 7.5, containing 0.75 M NaCl for 72 hours with changes at 24 and 48 hours. The other half was dialyzed in an identical manner except that 3.4 μg of [^{57}Co]vitamin-B_{12} were added to the protein-guanidine solution prior to dialysis. Each of the two dialyzed protein solutions was adjusted to a volume of 6.0 ml containing 10 mg of blue dextran 2000 and 2 mg of dinitrophenyl alanine and applied separately to the Sephadex G-150 column. In both of these experiments, a single symmetrical peak of vitamin B_{12}-binding activity (or [^{57}Co]vitamin-B_{12}) was observed at an elution position corresponding to a molecular weight of 38,000 (see Fig. 6). These two results suggest the possibility that the transcobalamin II subunits were not associated under the conditions in which these experiments were performed and that the 38,000 molecular weight subunit contains the binding site for vitamin B_{12}. It is also possible that transcobalamin II interacts with Sephadex at low protein concentrations with a resulting retardation of the protein.

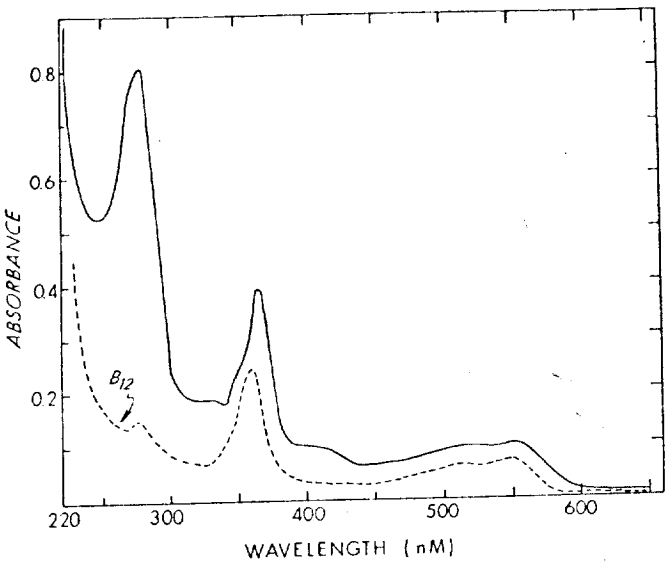

FIG. 7. Absorption spectra of the transcobalamin II-vitamin B_{12} complex and of unbound vitamin B_{12}. Spectra were obtained in 0.05 M potassium phosphate, pH 7.5, containing 0.75 M NaCl. The reference cuvette contained the same solution. ——, transcobalamin II (409 μg per ml) containing 11.7 μg per ml of bound vitamin B_{12}; – – –, vitamin B_{12} (11.7 μg per ml).

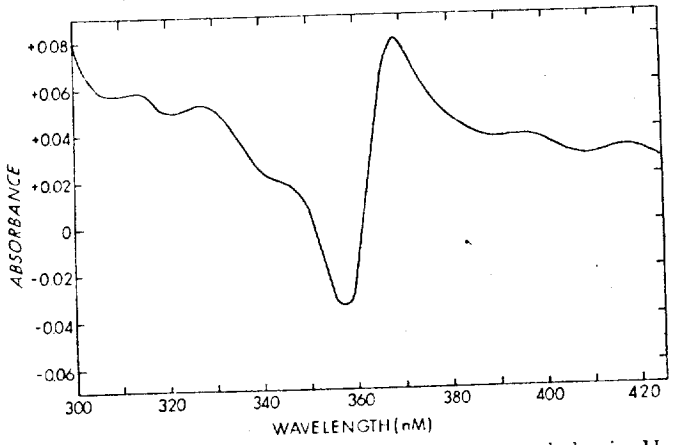

FIG. 8. Difference spectrum between the transcobalamin II-vitamin B_{12} complex and unbound vitamin B_{12} in 0.05 M potassium phosphate, pH 7.5, containing 0.75 M NaCl. The reference cuvette contained 17.9 μg per ml of unbound vitamin B_{12} and the second cuvette contained 11.7 μg per ml of vitamin B_{12} and 409 μg per ml of transcobalamin II.

Absorption and Difference Spectra—The spectrum of the transcobalamin II-vitamin B_{12} complex is presented in Fig. 7 together with the spectrum of an equal concentration of unbound vitamin B_{12}. When vitamin B_{12} is bound to transcobalamin II there appears to be a general enhancement of the vitamin B_{12} spectrum above 300 nm since the spectrum of transcobalamin II devoid of vitamin B_{12} in 7.5 M guanidine HCl, 0.05 M potassium phosphate, pH 7.5, is that of a typical protein with insufficient absorption above 300 nm to account for the difference between the two spectra presented in Fig. 7. When vitamin B_{12} binds to transcobalamin II, there is a shift in the 361 nm spectral maximum for unbound vitamin B_{12} to 364 nm for the transcobalamin II-vitamin B_{12} complex. The difference spectrum between the transcobalamin II-vitamin B_{12} complex and a concentration of unbound vitamin B_{12} containing equal absorption at 361 nm is presented in Fig. 8.

DISCUSSION

Transcobalamin II has been isolated in homogeneous form for the first time after being purified 2 million-fold relative to human plasma. Affinity chromatography on vitamin B_{12}-Sepharose was the crucial purification technique employed and resulted in a 24,000-fold purification of transcobalamin II. The fact that this technique has been applicable to the isolation of the granulocyte vitamin B_{12}-binding protein (14) as well as transcobalamin II suggests that it may be of general value in isolating other vitamin B_{12}-binding proteins.

Plasma fractions containing transcobalamin II facilitate the uptake of vitamin B_{12} by a number of different types of cells (9–12). The availability of homogeneous transcobalamin II allows for new experiments to elucidate the mechanism of protein facilitated cellular uptake of vitamin B_{12}. Preliminary experiments[1] indicate that our final preparation of transcobalamin II does facilitate vitamin B_{12} uptake by confluent cultures of human diploid fibroblasts. Thus, vitamin B_{12} bound to transcobalamin II is taken up by fibroblasts in significantly greater amount than either unbound vitamin B_{12} or vitamin B_{12} bound to the granulocyte vitamin B_{12}-binding protein. This result indicates that our final preparation of homogeneous transcobalamin II retains its functional ability as well as its ability to bind vitamin B_{12}.

Studies using crude preparations of transcobalamin II have indicated that this protein contains a single vitamin B_{12}-binding site (16) and has a molecular weight of 36,000 to 38,000 (2, 17) when determined by gel filtration. Our studies using homogeneous transcobalamin II also indicate that the protein has a single vitamin B_{12}-binding site, but our studies demonstrate a molecular weight of approximately 60,000 when measured by gel filtration, sedimentation equilibrium ultracentrifugation, and amino acid analysis. We have determined that transcobalamin II is a dimer consisting of 1 approximately 38,000 molecular weight subunit and 1 approximately 25,000 molecular weight subunit. Additional gel filtration experiments suggest that the 2 subunits may dissociate under certain conditions or that the protein interacts with Sephadex thus resulting in an apparent molecular weight of 38,000 based on the elution profile of protein-bound vitamin B_{12}. It is of interest that studies using crude transcobalamin II yield gel filtration molecular weight values greater than 38,000 for this protein under certain conditions (18–20) and that partial purification or high salt concentrations, or both, are required to obtain transcobalamin II in its 36,000 to 38,000 molecular weight form (2).

Transcobalamin II has a number of properties in common with the granulocyte vitamin B_{12}-binding protein (14), but the two proteins also differ in a number of respects. Similar properties include: (*a*) both proteins appear to have single vitamin B_{12}-binding sites and have molecular weights close to 60,000. (*b*) The presence of vitamin B_{12} is required to obtain maximal vitamin B_{12}-binding activity when the proteins are renatured from 7.5 M guanidine HCl. (*c*) Sulfhydryl compounds are deleterious to the renaturation of both proteins. (*d*) Neither protein contains any demonstrable free sulfhydryl groups. (*e*) When vitamin B_{12} is bound to either protein there is a general enhancement of the vitamin B_{12} spectrum above 300 nm.

Differences between transcobalamin II and the granulocyte vitamin B_{12}-binding protein include: (*a*) transcobalamin II contains one 38,000 molecular weight subunit and one 25,000 molecular weight subunit, whereas the granulocyte vitamin B_{12}-binding

[1] Unpublished experiments performed in collaboration with Miss Anne Lilljeqvist and Dr. Leon Rosenberg of Yale University.

appears to consist of a single polypeptide chain. (*b*) Transcobalamin II is eluted from vitamin B_{12}-Sepharose at lower concentrations of guanidine HCl and more rapidly than is the granulocyte protein. (*c*) Transcobalamin II is not a glycoprotein, whereas the granulocyte vitamin B_{12}-binding protein contains 33% carbohydrate. (*d*) The amino acid compositions of the two proteins are very different with major differences in their content of histidine, arginine, proline, alanine, leucine, and methionine. (*e*) When vitamin B_{12} is bound to transcobalamin II the 361 nm spectral peak for unbound vitamin B_{12} shifts to 364 nm. No shift occurs when vitamin B_{12} is bound to the granulocyte vitamin B_{12}-binding protein. (*f*) The difference spectra between the individual protein-vitamin B_{12} complexes and unbound vitamin B_{12} are quite different and suggest that the vitamin B_{12}-binding sites for the two proteins are not the same. (*g*) Transcobalamin II appears to facilitate the uptake of vitamin B_{12} by human diploid fibroblasts, whereas the granulocyte vitamin B_{12}-binding protein does not.

The differences between the amino acid and carbohydrate compositions of transcobalamin II and the granulocyte vitamin B_{12}-binding protein are compatible with the immunological differences that have been observed (2). We have previously summarized the immunological and other similarities between the granulocyte vitamin B_{12}-binding protein and transcobalamin I (14), and, on the basis of the differences between the former protein and transcobalamin II, it appears very unlikely that transcobalamin I and transcobalamin II are structurally related or that transcobalamin II is converted to transcobalamin I as has been suggested (12, 21).

Puutula and Gräsbeck (22) have recently purified transcobalamin II to the point where only approximately 60% to 70% noncobalamin B_{12}-binding protein was present. Only 60 μg of protein were obtained because of the low yield concomitant with a long series of conventional purification techniques. Despite this small amount of protein a number of physical studies were performed and several of these demonstrated different results than we have obtained.

Using the phenol sulfuric acid method (23) they obtained a 13.6% neutral hexose content for their final preparation of transcobalamin II. We have detected no carbohydrate residues using larger quantities of protein for analyses that have included amino acid analysis for amino sugars and a gas-liquid chromatographic method of carbohydrate analysis as well as the phenol sulfuric acid method. The most likely explanations for this discrepancy are that the 60% to 70% contaminating protein present in the final preparation of Puutula and Gräsbeck contained carbohydrate or that small fragments of Sephadex were present in their final preparation since gel filtration was used extensively in their purification scheme.

Puutula and Gräsbeck obtained a molecular weight for the transcobalamin II-vitamin B_{12} complex of 26,000 to 30,000 by sedimentation equilibrium ultracentrifugation in which the cells were scanned only at A_{280}. We obtained a molecular weight of 31,900 using the same technique and obtained similar values regardless of whether the cells were scanned at A_{280} or A_{360}. There are several possible explanations for the molecular weight discrepancy and these include: (*a*) the 60% to 70% contaminating protein present in the final preparation of Puutula and Gräsbeck is responsible since their cells were scanned only at A_{280}. (*b*) The 2 transcobalamin II subunits that we have demonstrated may not have been associated during their sedimentation equilibrium experiments. (*c*) If one of the 2 transcobalamin II subunits is capable of binding vitamin B_{12} alone, then Puutula and Gräsbeck may have isolated this subunit alone.

The latter possibility could conceivably also account for the third difference between their work and ours which concerns the fact that they did not observe a spectral shift in the vitamin B_{12} peak at 361 nm when the vitamin is bound to transcobalamin II, whereas we observed a shift to 364 nm. It is possible that the presence of both transcobalamin II subunits is required for the 361 nm → 364 nm shift and that this would not be observed if only 1 subunit was present. This question should be resolved when we complete our attempt to isolate the 2 subunits separately and study the role of each subunit in vitamin B_{12} binding and the effect that each subunit has on the vitamin B_{12} spectrum.

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A Study of Vitamin B_{12} Requirements in a Patient With Pernicious Anemia and Thyrotoxicosis: Evidence of an Increased Need for Vitamin B_{12} in the Presence of Hyperthyroidism

By JACK B. ALPERIN, MARY E. HAGGARD AND THOMAS P. HAYNIE

The response to small doses of vitamin B_{12} was studied in a woman with pernicious anemia and thyrotoxicosis. No response to 1 μg. per day occurred, but she did respond to 10 μg. per day. Fourteen months later when she was euthyroid, the patient did respond to 1 μg. per day. In another study, serum vitamin B_{12} levels were significantly lower in a group of patients with hyperthyroidism than in a group of normal subjects. Seventeen of 20 patients with hyperthyroidism exhibited significantly higher serum vitamin B_{12} levels 5–8 months after becoming euthyroid. The results of these studies provide evidence for increased vitamin B_{12} requirements in the presence of hyperthyroidism. The greater need for vitamin B_{12} appears to be related to increased utilization and/or accelerated turnover imposed by increased metabolism.

THE MINIMUM DAILY REQUIREMENT for vitamin B_{12} probably lies between 0.1 and 1.0 μg.;[1-4] however, 1 μg. per day is recommended for therapeutic trials in patients with vitamin B_{12} deficiency.[3,4] Numerous studies suggest an increased requirement for vitamin B_{12} in animals with hyperthyroidism.[5-11] There are several reports of patients with pernicious anemia and thyrotoxicosis;[13-15] however, none clearly shows an increased requirement for vitamin B_{12} in hyperthyroidism. Our discovery of a woman with pernicious anemia and thyrotoxicosis provided a unique opportunity to evaluate her vitamin B_{12} requirements before and after therapy with ^{131}I. We describe here the investigations performed on this patient and the results of serum vitamin B_{12} measurements in 34 other patients with hyperthyroidism.

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Methods

The following laboratory tests were performed with established methods: hemograms;[16] lobe average;[17] serum levels of folic acid,[18] vitamin B_{12},[19] iron,[20] lactic acid dehydrogenase (LDH),[21] and protein bound iodine (PBI);[22] resin sponge uptake of ^{131}I labeled triiodothyronine;[23] and uptake of ^{131}I by the thyroid gland.[24] Following the ingestion of 15 Gm. *l*-histidine monohydrochloride monohydrate, forminoglutamic acid (FiGlu) in urine was quantitated by a modified[25] electrophoretic technique.[26] The accompanying table gives normal values for these tests.

The acid content of gastric secretions was measured after maximal histamine stimulation[27] and peroral biopsy of the gastric mucosa was accomplished with the instrument described by Crosby and Kugler.[28] Vitamin B_{12} absorption was evaluated with a modified Schilling test[29] in which the patient ingests 0.5 μg. cyanocobalamin (CN-B_{12}) labeled with 0.6 μCi ^{57}Co. Normally, over 6 per cent of the ingested radioactivity is flushed into the urine in 24 hours.

Case Report

First Admission

R.Y., a 67-year-old woman, entered the hospital complaining of weakness, heat intolerance and paresthesias in her hands and feet. An enlarged thyroid gland had been present for many years. Her appetite was poor and she had lost nearly 7 Kg. in the past 4 months. Blood pressure, respiratory rate and temperature were normal. The pulse was 118 beats per minute. Pallor of the skin and mucosal surfaces, lingual atrophy, widening of the palpebral fissure and lid lag were evident. The thyroid gland was diffusely enlarged and estimated to be three–four times normal size. A holosystolic murmur was heard over the entire precordium and a loud bruit was audible over the thyroid gland. Her spleen was palpable 3 cm. below the costal margin. Diminished vibratory sensation (C 256 tuning fork) in both ankles and wrists was evident; otherwise, no abnormalities were detected on neurological ex-

Table 1.—Results of Laboratory Studies

	First Admission*	First Outpatient Visit †	Second Outpatient Visit ‡	Second Admission §	Normal Values
Hemoglobin (Gm. %)	8.8	12.3	10.3	9.2	
Red cells (10^6/cu. mm)	2.64	4.31	3.52	3.10	
Hematocrit (%)	27	49	34	31	
MCV (μ^3)	103	93	96	100	
MCH (pg.)	34	26	29	30	
Reticulocytes (%)	1.2	0.4	0.9	0.2	
Platelets (10^3/cu. mm)	98	342	189	100	
Leukocytes (10^3/cu. mm)	2.8	9.6	6.3	4.8	
Lobe average	5.1	3.3	4.1	4.9	3.1–3.5
Serum folate (ng./ml.)	12.4			6.8	3–14
Serum vitamin B_{12} (pg./ml.)	54		98	68	200–900
FiGlu (mg./12 hours)	298			83	< 25
LDH (mU./ml.)	2932			824	80–200
Serum Fe (μg. %)	210			142	75–140
PBI (μg. %)	18.7	6.4	4.8	3.9	3–8
T_3 resin sponge uptake (%)	40.3		27.6	24.8	25–35
24 hour thyroid uptake of ^{131}I (%)	72			12	10–40

* May 1964.
† Oct. 13, 1964.
‡ June 6, 1965.
§ Aug. 1965.

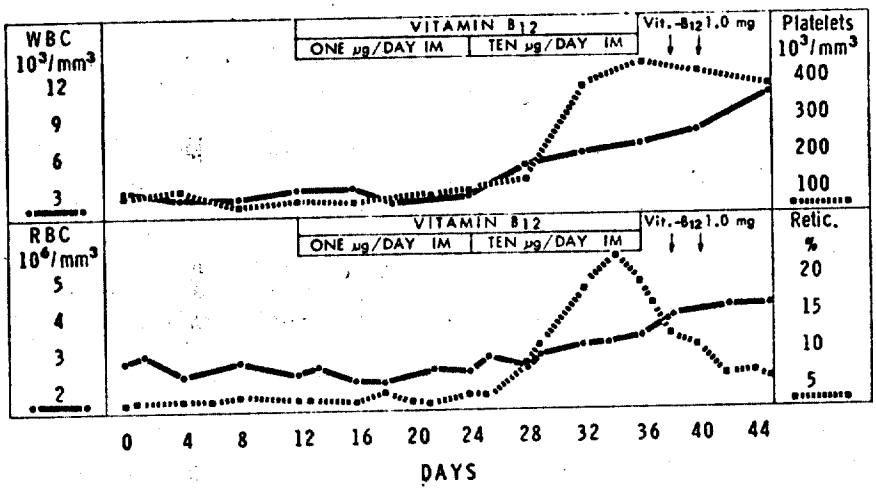

Fig. 1.—Hematological response to small quantities of CN-B_{12} when the patient had hyperthyroidism.

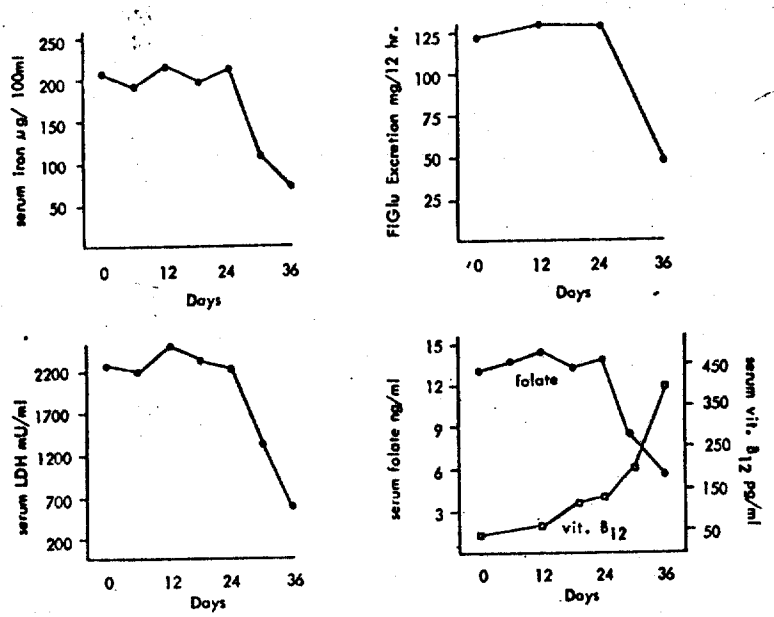

Fig. 2.—Biochemical changes in response to vitamin B_{12} supplements when the patient had hyperthyroidism. No vitamin B_{12} supplement was given during the initial 12-day period of observation. From day 13 through day 24 the patient received 1 µg. vitamin B_{12} per day; from day 25 through day 36 she received 10 uµ. per day.

amination. Results of important laboratory studies performed within 72 hours after admission appear in Table 1. A Wright's stained blood smear revealed many macroovalocytic red cells and hypersegmented neutrophilic leukocytes. Kingsley's stained[29] bone marrow smears disclosed typical megaloblastic changes. Chest X-ray showed moderate cardiac enlargement; ECG revealed a sinus tachycardia. Tests of renal and hepatic function were normal. Tests for malabsorption syndromes were negative.

A diagnosis of vitamin B_{12} deficiency and thyrotoxicosis was rendered. Before treating the

patient with ^{131}I, we observed her response to small, titrated doses of CN-B_{12} (Figs. 1 and 2). Finally before leaving the hospital, she was given two larger injections of CN-B_{12}, each consisting of 1000 μg. No additional vitamin B_{12} was administered until the second admission.

Interium Examinations

Four months after discharge, the patient was examined again. She felt much stronger and appeared clinically euthyroid. Her weight had increased almost 5 Kg. Neurological examination disclosed no abnormalities; the tip of the spleen was still palpable. Eight months later (1 year after leaving the hospital) she continued to feel well and appear euthyroid. Because of recurrent anemia (see Table 1), hospitalization was advised. She refused and another 2 months elapsed before she was readmitted.

Second Admission

At the time of the second hospital admission, her only complaints were weakness and paresthesias in her hands and feet of approximately 4 weeks duration. All vital signs were normal. Enlargement of the thyroid gland was no longer evident. Lingual atrophy and splenomegaly persisted. Absent vibratory sensation (C 256 tuning fork) was evident in her ankles and wrists, but no other neurological abnormalities were noted. Laboratory data obtained at the beginning of this admission appear in Table 1. The bone marrow again revealed megaloblastic erythropoiesis. Clinical and laboratory data showed the patient was now euthyroid, but vitamin B_{12} deficiency remained. Once more her response to a small dose of CN-B_{12} was determined (Figs. 3 and 4).

Results

Hematological Data

During both hospitalizations, the patient ate a standard diet calculated to contain about 20 μg vitamin B_{12} per day.[31] Figure 1 indicates the hematological response to CN-B_{12} while the patient had hyperthyroidism. During the initial 12 days of this study, supplemental vitamin B_{12} was not permitted and no improvement was seen. Similarly, no significant improvement appeared after

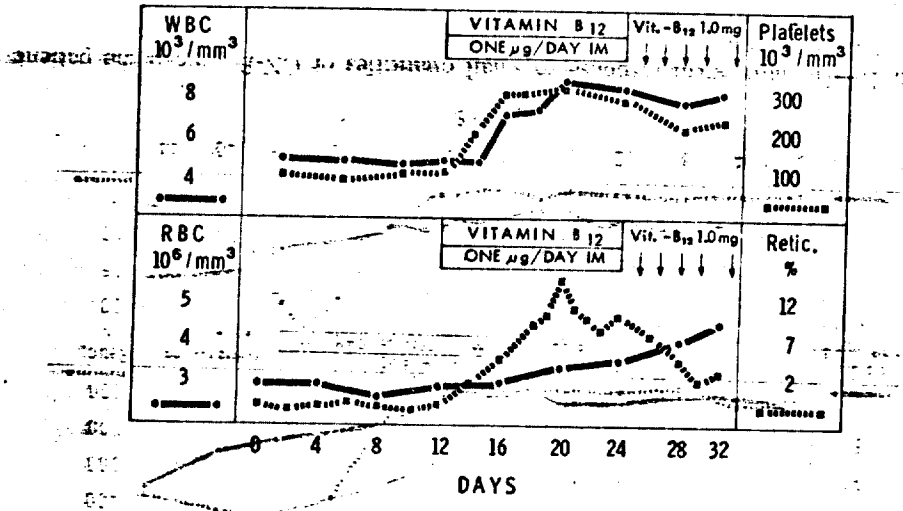

Fig. 3.—Hematological response to small quantities of CN-B_{12} when the patient was euthyroid.

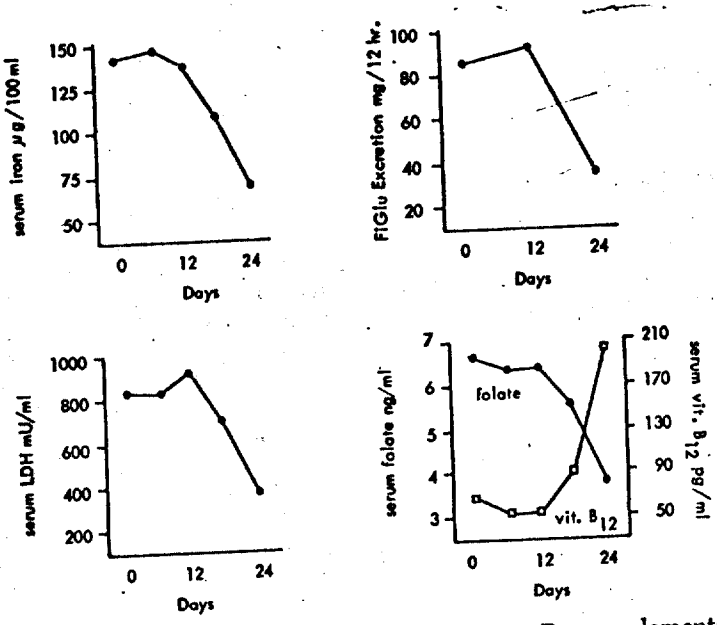

Fig. 4.—Biochemical changes in response to vitamin B_{12} supplements when the patient was euthyroid. No vitamin B_{12} supplement was allowed during the initial 12-day period of observation; however, from day 13 through day 24 she received 1 μg per day.

a daily injection of 1 μg. CN-B_{12}* for 12 days. When the daily injection of CN-B_{12} was increased to 10 μg, a brisk reticulocytosis ensued attaining a peak of 23.3 per cent on day 34 of the study. A rise in platelets and leukocytes also occurred with the larger dose of CN-B_{12}. Typical megaloblastic changes were observed in the bone marrow at the beginning of this study and again on day 24. By the 36th day, megaloblasts were no longer evident in the bone marrow; instead only an intense normoblastic erythroid hyperplasia appeared.

Hematological response to vitamin B_{12} therapy when the patient was euthyroid appears in Fig. 3. Once again, diet alone produced no response; however, the reticulocyte count quickly climbed to 15.3 per cent when the patient was given daily injections of 1 μg. CN-B_{12}. Leukocyte and platelet counts also increased significantly. On the first and twelfth days of this study, the bone marrow was megaloblastic. By day 24, the marrow revealed only normoblastic hyperplasia.

Biochemical Data

Results of serial biochemical determinations appear in Figs. 2 and 4. Concentrations of iron, LDH and folate in serum and the urinary excretion of FiGlu remained elevated during each initial 12-day period of observation. When the patient was hyperthyroid, 1 μg. CN-B_{12} per day for 12 days failed to significantly alter these parameters, but 10 μg. per day produced a significant decline in each. After the patient became euthyroid, a prompt fall in the levels

*The 1 and 10 μg. doses of CN-B_{12} referred to in this report were quantitated by assay with *Euglena gracilis*.[19]

of iron, folate, FiGlu and LDH followed treatment with just 1 μg CN-B_{12} per day. While the patient had hyperthyroidism, 1 μg of CN-B_{12} daily failed to substantially elevate the serum vitamin B_{12} level; however, a significant increase in the serum level occurred with 10 μg. per day. After the patient became euthyroid, the 1 μg. dose of CN-B_{12} caused a significant increase in the serum vitamin B_{12} level.

Studies of Thyroid Function

Thyroid function tests obtained at the beginning of the first hospitalization were diagnostic of hyperthyroidism (Table 1) and remained so until day 42 when the patient was treated with 5 mCi ^{131}I. Subsequently, she appeared clinically euthyroid and tests of thyroid function performed during two clinic visits and during the second hospitalization were within normal limits.

Examinations of the Stomach and Measurements of Vitamin B_{12} Absorption

During each hospitalization, the patient exhibited histamine-fast achlorhydria and peroral biopsy specimens of the gastric mucosa showed atrophic gastritis. Roentgenographic studies showed no abnormalities of the esophagus, stomach or small intestine. The Schilling test performed at the end of the first hospitalization revealed 1.1 per cent excretion of radioactivity without intrinsic factor and 24.2 per cent excretion with intrinsic factor. A repeat Schilling test during her second hospitalization gave almost identical results, i.e., without intrinsic factor 0.8 per cent excretion and with intrinsic factor 17.7 per cent excretion.

B_{12} Measurements in Patients with Hyperthyroidism

Serum vitamin B_{12} levels were measured in 34 patients with thyrotoxicosis which by history had been present at least 9 months. Each exhibited normal red cell morphology, a normal lobe average and normal leukocyte and platelet counts. Hemoglobin values varied from 9.8 to 13.1 Gm. per 100 ml. Thirty-two patients had normal serum vitamin B_{12} levels; one had a level of 180 pg. per ml. and the other, a level of 192 pg. per ml. The mean serum vitamin B_{12} level for all patients with hyperthyroidism was 347 $\pm$ 144 pg. per ml. In a group of 54 normal subjects, the mean serum vitamin B_{12} value was 572 $\pm$ 183 pg. per ml. Thus, vitamin B_{12} levels were significantly lower in the hyperthyroid group than in the normal group ($p < 0.01$).

Each patient was treated with ^{131}I. A second measurement of serum vitamin B_{12} activity was performed in 20 patients 5–8 months after they became euthyroid. None received supplemental vitamin B_{12} during the period of observation. All but three experienced an increase in the vitamin B_{12} level; two had decreased values; and the third exhibited no significant change. The two patients whose original vitamin B_{12} levels were $\leq$200 pg. per ml. exhibited normal levels after they became euthyroid. The mean vitamin B_{12} level in these 20 patients before therapy with ^{131}I was 335 $\pm$ 144 pg. per ml. After they became euthyroid, the mean serum vitamin B_{12} level measured 596 $\pm$ 171 pg. per ml. This difference represents a significant improvement ($p < 0.01$).

Discussion

Vitamin B_{12} supplements may protect rats against weight loss and death from thyrotoxicosis.[5-11] Furthermore, a number of metabolic and biochemical abnormalities occur in hyperthyroid rats which are corrected or prevented by treatment with vitamin B_{12}. These abnormalities include uncoupling oxidative phosphorylation of mitochondria,[9] decrease in serum proteins,[9,10] rapid loss of vitamin B_{12} and glutathione from the blood and liver,[10,11] decline in total content of soluble sulfhydryl-containing compounds in the liver[11] and an increase in the hepatic content of lipid and coenzyme A.[11] Methionine and betaine, given in place of vitamin B_{12}, may also protect against toxic effects of hyperthyroidism.[8,11] Both methionine and betaine have labile methyl groups and are important sources of one carbon fragments for intermediary metabolism.[32] Since vitamin B_{12} plays a major role in the transport of one carbon moieties,[32,33] it is tempting to speculate that thyrotoxicosis may also lead to a defect in the metabolism of one carbon fragments.

Each of 10 euthyroid patients with pernicious anemia that we studied[34] exhibited an excellent clinical and laboratory response to 1 μg. CN-B_{12} per day, in keeping with previous observations.[3,4] In the case reported herein, a woman with hyperthyroidism and pernicious anemia was treated with small doses of CN-B_{12}. While her hyperthyroidism remained untreated, no hematological or biochemical response to 1 μg. CN-B_{12} per day occurred; however, response to 10 μg. per day was excellent. After she became euthyroid, she exhibited a satisfactory response to 1 μg. CN-B_{12} per day. These studies clearly indicate she needed more CN-B_{12} when she had hyperthyroidism. The body can not directly utilize CN-B_{12}. It must first be converted to metabolically active forms, i.e., deoxyadenosyl-B_{12}, methyl-B_{12}, hydroxy-B_{12}, etc.[32,33] The possibility that hyperthyroidism in this patient interfered with the conversion of CN-B_{12} into metabolically active forms was not excluded, but seems unlikely. More likely she required more CN-B_{12} because vitamin B_{12} utilization and/or turnover accelerates in the presence of hyperthyroidism. Large amounts of vitamin B_{12} are presumably needed to satisfy increased metabolic needs which occur in hyperthyroidism.

Ziffer et al.[35] found whole blood vitamin B_{12} levels before and after an injection of 50 μg. of vitamin B_{12} significantly lower in patients with hyperthyroidism than in euthyroid subjects. Also, the urinary excretion of vitamin B_{12} after this injection was considerably less in the hyperthyroid group. The 34 patients with hyperthyroidism we studied showed significantly lower serum vitamin B_{12} levels than a group of normal men and women. Furthermore, 17 of 20 patients with hyperthyroidism exhibited significant increases in serum vitamin B_{12} levels after they became euthyroid. These data offer further evidence for an increased requirement for vitamin B_{12} in the presence of hyperthyroidism.

Decreased hepatic stores of folic acid in hyperthyroid rats[36] and rapid clearance of intravenously injected folic acid in patients with thyrotoxicosis[37] suggest that folic acid requirements are also increased in hyperthyroidism. Needed are reports of patients with hyperthyroidism and megaloblastic

anemia due to folic acid deficiency. Two patients with thyroid storm, folic acid deficiency and megaloblastic bone marrow have been examined in our laboratory; however, they were too ill to permit evaluation of their response to small doses of folic acid.[34]

Scores of investigations show a definite relationship between pernicious anemia and hyperthyroidism. A scholarly review of this subject appeared in a recently published textbook.[12] There is an increased frequency of pernicious anemia in patients with hyperthyroidism. Conversely, patients with pernicious anemia have a higher incidence of hyperthyroidism. In a few instances, both diseases have been discovered simultaneously[13-15] such as the patient described in this report. Further evidence for an association between pernicious anemia and thyrotoxicosis derives from determinations of autoantibodies. Parietal cell antibodies may be detected in the serum of about 85 per cent of patients with pernicious anemia and nearly 30 per cent of patients with hyperthyroidism. Thyroid antibodies in serum may be found in over half the patients with pernicious anemia and about 45 per cent of patients with hyperthyroidism. The incidence of these two antibodies in control sera does not exceed 15 per cent. About one-half of patients with pernicious anemia have intrinsic factor antibodies in serum, and pernicious anemia has been present in most instances wherein intrinsic factor antibodies were detected in patients with hyperthyroidism.[12,38] A search for intrinsic factor and thyroid antibodies in our patient produced only negative results; we did not measure parietal cell antibodies.

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§ 3.32 Label declarations of vitamin B_{12} and folic acid in foods for special dietary uses.

(a) Section 125.3(a)(2) of this chapter, issued under the authority of section 403(j) of the Federal Food, Drug, and Cosmetic Act (sec. 403(j), 52 Stat. 1048; 21 U.S.C. 343(j)) requires that if a food purports to be or is represented for special dietary use by man by reason, in whole or in part, of a vitamin for which the need in human nutrition has not been established, the label of such food shall bear the statement "The need for _____ in human nutrition has not been established," the blank to be filled in with the name of such vitamin.

(b) Heretofore this Department has considered vitamin B_{12} and folic acid as among those vitamins for which the need in human nutrition has not been established. However, recent scientific evidence shows that these vitamins are needed in human nutrition. The Department therefore considers the requirement of § 125.3 (a) (2) quoted in paragraph (a) of this section as no longer applicable to food offered for special dietary use by reason of these vitamins.

(Sec. 403, 52 Stat. 1047, as amended; 21 U.S.C. 343) [20 F.R. 9549, Dec. 20, 1955]

§ 3.40 Preparations for the treatment of pernicious anemia.

(a) The ninth announcement of the Anti-anemia Preparations Advisory Board of the United States Pharmacopeia is concerned with the status of the treatment of pernicious anemia. It clearly presents the following facts:

(1) The Sixteenth Revision of the Pharmacopeia of the United States, which became official on October 1, 1960, does not include preparations intended for the treatment of pernicious anemia by oral administration.

(2) The U.S.P. unit for anti-anemia preparations no longer has any significance.

(3) The U.S.P. Anti-anemia Preparations Advisory Board was disbanded.

(b) On the basis of the scientific evidence and conclusions summarized in the statement of the U.S.P. Anti-anemia Preparations Advisory Board as well as pertinent information from other sources, the Commissioner of Food and Drugs finds it is the consensus of well informed medical opinion that:

(1) The parenteral administration of cyanocobalamin or vitamin B_{12} is generally recognized as a fully effective treatment of pernicious anemia. Parenteral cyanocobalamin preparations have not been and are not authorized for use except by or on the prescription of a duly licensed medical practitioner.

(2) Some patients afflicted with pernicious anemia do not respond to orally ingested products. There is no known way to predict which patients will fail to respond or will cease to respond to the treatment of pernicious anemia with orally ingested preparations.

(3) The substitution of a possibly inadequate treatment, such as the ingestion of oral preparations of vitamin B_{12} with intrinsic factor concentrate, in place of parenteral vitamin B_{12} products for a disease condition as serious as pernicious anemia cannot be regarded as safe in all cases.

(4) The development of the classical symptoms of pernicious anemia that would cause a person to seek medical attention may in some cases be delayed by oral ingestion of intrinsic factor. Pernicious anemia is a disease that is associated, among other things, with a higher than normal incidence of cancer of the stomach and that for the safety of the patient, requires continuous expert medical supervision.

(5) With inadequate treatment there may be markedly deleterious effects on the nervous system. It is well established that whereas the development of anemia is completely reversible with adequate treatment, the involvement of the nervous system may not be completely reversible and thus may result in permanent damage.

(6) Some hematologists prescribe oral preparations of vitamin B_{12} in the treatment of pernicious-anemia patients.

(7) Intrinsic factor and intrinsic factor concentrate serve no known useful therapeutic or nutritive purpose except to the extent that they do increase the gastrointestinal absorption of vitamin B_{12} in patients with a deficiency or absence of intrinsic factor, which may eventually lead to pernicious anemia. This conclusion does not apply to diagnostic procedures using radioactive cyanocobalamin.

(8) Medical expertise is required for the diagnosis as well as the management of pernicious anemia.

(c) The Eleventh Edition of The National Formulary and its first Interim Revision include monographs for oral preparations of vitamin B_{12} with intrinsic factor concentrate, establish a unit of vitamin B_{12} with intrinsic factor concentrate, and provide for a National Formulary Anti-anemia Preparations Advisory Board to assign the potency of such preparations. This provides for the availability of such oral preparations, standardized within the meaning of the broad limits characteristic of the evaluation of such preparations.

(d) Any drug that is offered for or purports to contain intrinsic factor or intrinsic factor concentrate will be regarded as misbranded within the meaning of section 503(b) of the Federal Food, Drug, and Cosmetic Act unless it is labeled with the legend "Caution—Federal law prohibits dispensing without prescription."

(e) Any drug for oral ingestion intended, represented, or advertised for the prevention or treatment of pernicious anemia or which purports to contain any substance or mixture of substances described in paragraph (d) of this section (other than diagnostic drugs containing radioactive cyanocobalamin) will be regarded as misbranded under sections 502 (f)(2) and (j) of the act unless its labeling bears a statement to the effect that some patients afflicted with pernicious anemia may not respond to the orally ingested product and that there is no known way to predict which patients will respond or which patients may cease to respond to the orally ingested products. The labeling shall also bear a statement that periodic examinations and laboratory studies of pernicious-anemia patients are essential and recommended.

(f) Under section 409 of the Federal Food, Drug, and Cosmetic Act, intrinsic factor and intrinsic factor concentrate are regarded as food additives. No food additive regulation nor existing extension of the effective date of section 409 of the act authorizes these additives in foods, including foods for special dietary uses. Any food containing added intrinsic factor or intrinsic factor concentrate will be regarded as adulterated within the meaning of section 402(a)(2)(C) of the act.

(g) Regulatory action may be initiated with respect to any article shipped within the jurisdiction of the act contrary to the provisions of this policy statement after the 180th day following publication of this statement in the FEDERAL REGISTER.

(Secs. 402, 502, 503, 52 Stat. 1051, 1052 as amended; 65 Stat. 648, 72 Stat. 1784; 21 U.S.C. 342, 352, 353) [28 F.R. 1586, Feb. 20, 1963]

§ 3.42 Status of pteroylglutamic acid (folic acid) in foods for special dietary use and as a drug.

(a) Pteroylglutamic acid (folic acid) for food use is regarded as a food additive, subject to the provisions of section 409 of the Federal Food, Drug, and Cosmetic Act. As provided by § 121.1134 of this chapter which became effective July 20, 1963, pteroylglutamic acid (folic acid) may be safely used as a component of dietary supplements, provided the directions for use are such that, when followed, daily ingestion will not exceed 0.1 milligram of the additive.

(b) Preparations supplying over 0.1 milligram of pteroylglutamic acid (folic acid) in the daily dosage recommended or suggested may be marketed only as drugs for use under medical supervision. Among other things, these drugs must be labeled with the statement "Caution: Federal law prohibits dispensing without prescription," and their labeling must bear adequate information for their use by physicians, including information about the possible harmful effect of administering pteroylglutamic acid (folic acid) to patients who may have pernicious anemia.

(c) Because preparations represented or intended for drug use and supplying 0.1 milligram or less of pteroylglutamic acid (folic acid) in the daily dosage recommended or suggested may also supply other active ingredients, each such preparation should be considered on an individual basis to establish its status under the Federal Food, Drug, and Cosmetic Act.

(Secs. 403(j), 409, 502(f), (j), 52 Stat. 1048, 1051; 72 Stat. 1785; 21 U.S.C. 343(j), 348, 352(f), (j)) [29 F.R. 2557, Feb. 19, 1964]

§ 3.48 Cobalt preparations intended for use by man.

(a) On January 17, 1967 (21 CFR 3.48; 32 F.R. 449), the Commissioner of Food and Drugs issued a revised statement of policy with respect to the status of cobalt-containing drug preparations intended for use by man, which revision was to be modified as needed following consideration of such drugs by a panel of hematologists. A panel consisting of authorities in the field of hematology met on March 8, 1967, with representatives of the Medical Advisory Board for the Food and Drug Administration to consider the status of cobalt-containing drugs and the following findings and recommendations were made:

(1) Cobalt salts are not suitable for over-the-counter sale to the public for the treatment of iron-deficiency anemia. They are associated with toxic effects and offer no advantage over iron alone.

(2) Potential toxic effects of these salts includes liver damage, claudication, myocardial damage, thyroid hyperplasia, hypothyroidism, dermatitis, nausea, and anorexia.

(3) Cobalt salts are not generally recognized as safe or effective therapy for any disease condition.

(b) On the basis of the available evidence and the findings and recommendations of the representatives of the Medical Advisory Board, the Commissioner of Food and Drugs finds and determines with respect to cobalt-containing drug preparations intended for use by man, except radioactive forms of

components named in paragraph (a) of this section.

(c) If it is necessary to add any nutrient(s) in order to meet the minimum nutrient levels prescribed in paragraph (d) of this section, the addition of each such nutrient may not result in a total nutrient level exceeding 150 percent of the minimum level prescribed. Nutrients used for such addition shall be biologically available in the final product.

(d) Minimum levels of nutrients for a frozen "heat and serve" dinner are as follows:

Nutrient	Minimum levels for frozen "heat and serve" dinner— for each 100 Calories (kcal) of the total components specified in paragraph (a)	for the total components specified in paragraph (a)
Protein, grams	4.60	16.0
Vitamin A, IU	150.00	520.0
Thiamine, mg	0.05	0.2
Riboflavin, mg	0.06	0.2
Niacin, mg	0.99	3.4
Pantothenic acid, mg	0.32	1.1
Vitamin, B_6, mg	0.15	0.5
Vitamin, B_{12}, mcg	0.33	1.1
Iron, mg	0.62	2.2

Subpart B—Nutritional Quality Guidelines

§ 100.5 Frozen "heat and serve" dinner.

(a) A product, for which a common or usual name is established in § 102.11 of this chapter, in order to be eligible to bear the guideline statement set forth at § 100.1(b), shall contain at least the following three components:

(1) One or more sources of protein derived from meat, poultry, fish, cheese, or eggs.

(2) One or more vegetables or vegetable mixtures other than potatoes, rice, or cereal-based product.

(3) Potatoes, rice, or cereal-based product (other than bread or rolls) or another vegetable or vegetable mixture.

(b) The three or more components named in paragraph (a) of this section, including their sauces, gravies, breading, etc.:

(1) Shall contribute not less than the minimum levels of nutrients prescribed in paragraph (d) of this section.

(2) Shall be selected so that one or more of the listed protein sources of paragraph (a)(1) of this section, excluding their sauces, gravies, breading, etc., shall provide not less than 70 percent of the total protein supplied by the

(1) A frozen "heat and serve" dinner prepared from conventional food ingredients listed in paragraph (a) of this section will also contain folic acid, magnesium, iodine, calcium, and zinc. Minimum levels for these nutrients cannot be established at the present time but may be specified as additional data are obtained.

(2) The minimum levels for pantothenic acid, vitamin B-6, and vitamin B-12 are tentative. Final levels will be established when sufficient data are available. Until final levels are established, a product containing less than the tentative levels will not be deemed to be misbranded when labeled in accordance with § 100.1(b).

(3) When technologically practicable iodized salt shall be used or iodine shall be present at a level equivalent to that which would be present if iodized salt were used in the manufacture of the product.

(4) When technologically practicable, product components and ingredients shall be selected to obtain the desirable calcium to phosphorous ratio of 1:1. Technological addition of phosphates shall be minimized and shall not ex-

§ 121.101 Substances that are generally recognized as safe.

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(a) It is impracticable to list all substances that are generally recognized as safe for their intended use. However, by way of illustration, the Commissioner regards such common food ingredients as salt, pepper, sugar, vinegar, baking powder, and monosodium glutamate as safe for their intended use. The lists in paragraph (d) of this section include additional substances that, when used for the purposes indicated, in accordance with good manufacturing practice, are regarded by the Commissioner as generally recognized as safe for such uses.

(b) For the purposes of this section, good manufacturing practice shall be defined to include the following restrictions:

(1) The quantity of a substance added to food does not exceed the amount reasonably required to accomplish its intended physical, nutritional, or other technical effect in food; and

(2) The quantity of a substance that becomes a component of food as a result of its use in the manufacturing, processing, or packaging of food, and which is not intended to accomplish any physical or other technical effect in the food itself, shall be reduced to the extent reasonably possible.

(3) The substance is of appropriate food grade and is prepared and handled as a food ingredient. Upon request the Commissioner will offer an opinion, based on specifications and intended use, as to whether or not a particular grade or lot of the substance is of suitable purity for use in food and would generally be regarded as safe for the purpose intended, by experts qualified to evaluate its safety.

(c) The inclusion of substances in the list of nutrients does not constitute a finding on the part of the Department that the substance is useful as a supplement to the diet for humans.

(d) Substances that are generally recognized as safe for their intended use within the meaning of section 409 of the act are as follows:

Product	Tolerance	Limitations or restrictions
(5) NUTRIENTS AND/OR DIETARY SUPPLEMENTS [1]—con.		
*Manganese hypophosphite		
*Manganese sulfate		
*Manganous oxide		
*Mannitol	5 percent	In special dietary foods.
*Methionine		Animal feeds.
*Methionine hydroxy analog and its calcium salts.		Do.
Niacin		
Niacinamide		
D-Pantothenyl alcohol		
*Phenylalanine (L- and DL-forms)		
Potassium chloride		
*Potassium glycerophosphate		
Potassium iodide	0.01 percent	In table salt as a source of dietary iodine.
*Proline (L- and DL-forms)		
Pyridoxine hydrochloride		
Riboflavin		
Riboflavin-5-phosphate		
*Serine (L- and DL-forms)		
Sodium pantothenate		
Sodium phosphate (mono-, di-, tribasic).		
Sorbitol	7 percent	In foods for special dietary use.
Thiamine hydrochloride		
Thiamine mononitrate		
*Threonine (L- and DL-forms)		
Tocopherols		
α-Tocopherol acetate		
*Tryptophane (L- and DL-forms)		
*Tyrosine (L- and DL- forms)		
*Valine (L- and DL-forms)		
Vitamin A		
Vitamin A acetate		
Vitamin A palmitate		
Vitamin B_{12}		
Vitamin D_2		
Vitamin D_3		
*Zinc sulfate		
*Zinc gluconate		
*Zinc chloride		
*Zinc oxide		
*Zinc stearate (prepared from stearic acid free from chickedema factor).		

§ 125.5 Label statements relating to infant food.

(a) If a food (other than a dietary supplement of vitamins and/or minerals alone) purports to be or is represented for special dietary use for infants, the label shall bear, if such food is fabricated from two or more ingredients, the common or usual name of each ingredient, including spices, flavoring, and coloring.

(b) If such food, or any ingredient thereof, consists in whole or in part of plant or animal matter and the name of such food or ingredient does not clearly reveal the specific plant or animal which is its source, such name shall be so qualified as to reveal clearly the specific plant or animal that is such source.

(c) If such use of the food is by reason of its simulation of human milk or its suitability as a complete or partial substitute for human milk, the label shall also bear:

(1) A statement of the percent by weight or weight per unit volume of moisture, protein, fat, available carbohydrate, ash, and crude fiber contained in such food.

(2) A statement of the number of available kilocalories (in the case of food label statements, a kilocalorie is represented by the word "Calorie") supplied by a specified quantity of such food as customarily or usually prepared for consumption.

(3) A statement of the amount of each vitamin or mineral listed in subparagraph (5) of this paragraph and the amount of other added vitamin(s) and mineral(s) supplied by a specified quantity of such food as customarily or usually prepared for consumption.

(4) The statement "This product should not be used as the sole source of protein in the infant diet" if a quantity which supplies 100 available kilocalories of such food as customarily or usually prepared for consumption contains less than 1.8 grams of protein of a biological quality equivalent to that of casein, or if the amount and biological quality of protein per 100 available kilocalories of such food are such that the quality of protein expressed as a fraction of that of casein multiplied by the amount of protein in grams is less than 1.8, or if the biological quality of protein is less than 70 percent of that of casein.

(i) For the purpose of this subparagraph, the method for determining biological quality of protein shall be the method prescribed on page 800 (secs. 39.166–39.170) under "Biological Evaluation of Protein Quality—Official, Final Action" of "Official Methods of Analysis of the Association of Official Analytical Chemists," 11th edition (1970).

(ii) For the purpose of this subparagraph, the method for determining the amount of protein is to multiply by 6.25 the total nitrogen content in grams, as determined by the method described on page 16 (sec. 2.051) under "Improved Kjeldahl Methods for Nitrate-Free Samples—Official, Final Action" of "Official Methods of Analysis of the Association of Official Analytical Chemists," 11th edition (1970).

(5) If a quantity which supplies 100 available kilocalories of such food as customarily or usually prepared for consumption contains less than the following amounts of vitamins and minerals, a statement that an additional quantity of such vitamin(s) or mineral(s), as the case may be, should be supplied from other sources:

Vitamins and minerals	Unit of measurement	Minimum amounts
Vitamin A	U.S.P. units	250
Vitamin D	do	40
Vitamin E	International units	0.3
Ascorbic acid (vitamin C)	Milligrams	7.8
Thiamin (vitamin B_1)	do	0.025
Riboflavin (vitamin B_2)	do	0.06
Niacin [1]	Milligram equivalents	0.8
Vitamin B_6	Milligrams	0.035
Folacin	Micrograms	4
Pantothenic acid	Milligrams	0.3
Vitamin B_{12}	Micrograms	0.15
Calcium	Milligrams	50
Phosphorus	do	25
Magnesium	do	6
Iron	do	1
Iodine	Micrograms	5
Copper	Milligrams	0.06

[1] The generic term "niacin" includes niacin (nicotinic acid), niacinamide (nicotinamide), and 1 milligram equivalent for each 60 milligrams of tryptophan in the food.

When a statement prescribed by this subparagraph is required, it shall appear in immediate proximity to the statement for the appropriate vitamin or mineral required by paragraph (c)(3). The difference in quantity between the amount of vitamin(s) and mineral(s) supplied and the amount required by this subparagraph, expressed on the same basis, must also appear in the same statement.

(6) If such food contains fat at a level supplying less than 15 percent of the total available kilocalories, or linoleic acid (present as a glyceride) at a level supplying less than 2 percent of the total available kilocalories, a statement that an additional quantity of fat or linoleic acid (linoleate), as the case may be, should be supplied from other sources. The requirement of this subparagraph shall not apply to such food which purports to be or is represented for special dietary use by reason of a need for regulating the intake of fat.

(d) The provisions of paragraph (c) of this section shall not apply to whole milk (of cows) or evaporated milk except with respect to ascorbic acid, vitamin D, and iron under paragraph (c)(5) of this section.

(e) A food which purports to be or is represented for special dietary use solely as a food for infants by reason of its simulation of human milk or its suitability as a complete or partial substitute for human milk, and which complies with the provisions of this section, shall be exempt from the effective provisions of §§ 125.3, 125.4, and 125.6 of this Part 125. [36 F.R. 23555, Dec. 10, 1971; 36 F.R. 24792, Dec. 23, 1971]

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The Incorporation of Acetate-2-^{14}C and Mevalonate-2-^{14}C into Cholesterol During Vitamin B_{12} Deficiency (35393)

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Vitamin B_{12} and its coenzyme forms are essential components in various areas of intermediary metabolism but their role in cholesterol metabolism has not been clarified. Forbes and Patterson (1), Hsu and Chow (2), and Icayan and Chow (3) have presented evidence demonstrating that vitamin B_{12} is an important factor in cholesterol metabolism. The current research project was undertaken to study possible B_{12} involvement in cholesterol synthesis, the following questions being of particular interest: (a) At which steps in the cholesterol biosynthetic pathway might the presence of vitamin B_{12} be required, and (b) Is the effect of vitamin B_{12} deficiency limited to hepatic cholesterol metabolism or is it a general phenomenon?

Materials and Methods. Materials. Radiochemicals. Sodium acetate-2-^{14}C (sp act 8.56 mCi/mole) was purchased from Tracerlab-Keleket, Waltham, Massachusetts. Toluene-^{14}C standard and DL-mevalonic acid-2-^{14}C–lactone (sp act 3.35 mCi/mmole) were purchased from Nuclear-Chicago, Des Plaines, Illinois. Cholesterol purchased from Matheson Co. was recrystallized five times from absolute ethanol. PPO (2,5-diphenyloxazole) and POPOP [1,4-bis-2-(5-phenylaxazolyl) Benzene], scintillation grade, were obtained from Packard Instrument Company. All other chemicals were ACS grade.

Experimental animals. Five-month-old McCollum strain female rats were mated and then maintained on a 62% soybean protein diet (3), unsupplemented with cyanocobalamin throughout gestation and lactation. Pregnant rats were housed separately in raised, wide-mesh, screen-bottomed cages until day 19 of gestation at which time, they were transferred to cages containing bedding. To limit coprophagy, bedding was changed once a day and fecal pellets seen at any time were removed with forceps. A 28 day lactation period was used to ensure survival of progeny. At the end of that period, progeny were separated on the basis of sex, age, and body weight. Female offsprings were discarded while male progeny were divided into two groups, one receiving the basal diet without vitamin B_{12} and the other, the same diet containing 50 μg of B_{12}/kg of diet.

At the end of the B_{12} deprivation period, rats were fasted overnight but with water *ad libitum*. On the following morning, animals were weighed, given the radioactive form of a cholesterol precursor through intraperitoneal injection, and were housed in metabolism cages under an exhaust hood for the duration of the incorporation period. Subsequently, animals were sacrificed under light ether anesthesia by cardiac puncture. Blood, along with various tissues, was either processed immediately for assay or prepared for storage in a frozen state until used.

Methods. Quantitative and radiometric measurement of cholesterol. Cholesterol was isolated according to the method of Sperry and Webb (4) with volumes of reagents being adjusted to accommodate sample size. Precipitated cholesteryl digitonide was dissolved in 1.0 ml of glacial acetic acid at 100°. For colorimetric analysis, 0.1-ml aliquots were diluted with glacial acetic acid to a volume of 2.0 ml. The Liebermann-Burchard reaction was employed for color development and its absorbance was measured in a Spectronic 20 photometer at 625 mμ. Aliquots (0.2 and 0.4 ml) were used for radioactivity tests. Samples were added to 20-ml capacity low potassium counting vials and 10 ml of chilled Diatol scintillation solvent were dispensed into each vial. The

TABLE I. Liver and Plasma Cholesterol Content in B_{12} Deficient and Treated Rats.[a]

Group	Type of diet	Total	Free	Ester	F/T
		Liver (mg/g)			
A	B_{12} supplemented	2.46 ± 0.02	1.98 ± 0.04	0.60 ± 0.02	0.76 ± 0.03
B	B_{12} deficient	2.20 ± 0.01	1.82 ± 0.02	0.40 ± 0.02[b]	0.83 ± 0.02
		Plasma (mg/100 ml)			
A	B_{12} supplemented	111 ± 4.4	32 ± 6.0	79 ± 10.0	0.29 ± 0.05
B	B_{12} deficient	77 ± 12.0[b]	38 ± 3.0	39 ± 10.9[c]	0.49 ± 0.09[b]

[a] Each value represents an average of five, 8-month-old male rats. Results are given as mean ± SEM.

[b] Significant differences between B_{12} supplemented and B_{12} deficient groups are marked: $^b p < 0.05$; $^c p < 0.01$.

solvent system, Diatol, consisted of toluene, 350 ml; anhydrous methanol, 210 ml; recrystallized naphthalene, 73 g; dioxane, 350 ml; and the phosphors: PPO, 4.6 g and POPOP, 80 mg. Corrections for quench were made through the internal standard method. Samples were counted for 10-min intervals in the Tri-carb liquid scintillation counter, model 314AX. To estimate the counting efficiency, either sodium acetate-2-^{14}C or mevalonic acid-2-^{14}C–lactone standards were run as well as a commercial toluene-^{14}C standard. Total lipid determination: Soxhlet extraction was employed for the isolation of total lipid. Tissues were ground with anhydrous sodium sulfate, stored in a vacuum desiccator to dry overnight and on the next day, were extracted with chloroform for a period of 16 hr at 80°. Lipid extracts were brought to dryness through air evaporation and were stored in a desiccator until constant weight was reached. Total lipid was determined gravimetrically.

Results. Data in Table I show that in B_{12} deficient rats as compared with B_{12} treated, liver total cholesterol was lower by 15% and the ester fraction was diminished by 33% ($p<0.01$). Free cholesterol was not appreciably altered; the proportion of free to total cholesterol was comparable between the two groups. Plasma analysis revealed that total cholesterol was markedly decreased in B_{12} deficient rats, the difference between the two groups being 40%. Plasma free cholesterol was not altered but the ester fraction of the deficient rat was reduced by 50% ($p<0.05$). Further, the plasma free to total ratio was significantly higher for the B_{12} deficient group.

Data in Table II show that after 30 min, sp act of hepatic cholesterol in the deficient group was significantly higher than in the treated group ($p<0.05$). These findings were in agreement with those of Icayan and Chow (3). A significant increase in radioactivity per gram and in specific activity of cholesterol was also noted in B_{12} deficient rats 3 hr after

TABLE II. Incorporation of Acetate-2-^{14}C into Liver Cholesterol.[a]

Group	No. of rats	Incorporation period (min)	Cholesterol (mg/g of liver)	Radioactivity isolated CH (cpm/g of liver)	Sp act (cpm/mg of CH)
A	4	30	2.32 ± 0.15	1315 ± 322	562 ± 123
B	5	30	1.98 ± 0.12	1860 ± 180	971 ± 135[b]
A	6	180	2.56 ± 0.07	884 ± 199	350 ± 82
B	6	180	2.08 ± 0.06	1818 ± 296[b]	872 ± 192[b]

[a] Each rat received a single intraperitoneal injection of sodium acetate-2-^{14}C (5 μCi/0.2 mmole/100 g of body wt); and was killed by decapitation at indicated time intervals. Results are given as mean ± SEM.

[b] Significant differences between B_{12} supplemented and B_{12} deficient groups are marked: $p < 0.05$.

TABLE III. Incorporation of Mevalonic-^{14}C-Lactone into Liver Cholesterol by B_{12} Deficient and Treated Male Rats.[a]

Group	No. of rats	Type of diet	Incorporation period (min)	Cholesterol (mg/g of liver)	Radioactivity isolated CH (cpm/g of liver)	Sp act (cpm/mg of CH)
A	3	B_{12} supplemented	30	2.53 ± 0.1	10,009 ± 1424	3918 ± 223
B	2	B_{12} supplemented	30	2.16 ± 0.08	10,168 ± 164	4702 ± 264
C	6	B_{12} deficient	30	2.07 ± 0.17	7704 ± 1213	3724 ± 496
A	12	B_{12} supplemented	120	2.17 ± 0.04	8438 ± 379	3888 ± 106
C	14	B_{12} deficient	120	2.04 ± 0.05	7350 ± 384	3615 ± 143
A	3	B_{12} supplemented	240	2.59 ± 0.07	9619 ± 1139	3702 ± 283
B	3	B_{12} supplemented	240	2.45 ± 0.09	10,758 ± 1209	4372 ± 294
C	6	B_{12} deficient	240	2.26 ± 0.07	6092 ± 859[b]	2679 ± 328[c]

[a] Groups A and C were progeny of mothers fed the vitamin B_{12} deficient diet during pregnancy and lactation, and Group B was progeny of mothers fed Purina Lab Chow during pregnancy and lactation. Results are given as mean ± SEM.

[b] Significant differences between B_{12} deficient rats killed at 30 and 120 min and at 240 min are marked: $p < 0.05$.

[c] Significant differences between supplemented and deficient groups killed at 240 min are marked: $p < 0.05$.

isotope injection.

After 30 min and 2 hr the amount of the mevalonate radioactivity in the liver cholesterol shown in Table III was found to be the same in the B_{12} deficient and B_{12} supplemented rats. After 4 hr the concentration of radioactive cholesterol (cpm/g of liver and its sp act) decreased significantly in B_{12} deficient rats. Such changes were not observed in B_{12} supplemented animals.

To determine the effects of vitamin B_{12} on cholesterol synthesis in the organs other than the liver, the testes, adrenals, and brain were examined. Table IV shows that no observable difference in relative weights of the above 3 organs between B_{12} deficient and B_{12} supplemented rats. The total lipid and cholesterol contents in the adrenals of B_{12} deficient rats were markedly increased over those of B_{12} supplemented rats. Although differences were not noticed in radioactivity per gram of tissue, the sp act was significantly reduced in B_{12} deficient rats. Unlike adrenals, deficiency of B_{12} had no effect on brain cholesterol. In the testes, cholesterol content was unaffected by B_{12} deficiency. But the radioac-

TABLE IV. Cholesterol Content and Synthesis in Extrahepatic Tissues of Vitamin B_{12} Deficient and Treated Male Rats.

Group	Organ	Relative organ wt	Lipid (mg of CH/g)	Cholesterol (mg/g of tissue)	Radioactivity isolated CH (cpm/g of tissue)	Sp act (cpm/mg of CH)
A	Testes	0.95 ± 0.02	—	1.50 ± 0.13	207 ± 39	140 ± 8
B		1.05 ± 0.04		1.74 ± 0.08	120 ± 18	68 ± 8[b]
A	Adrenals	6.67 ± 0.41	46 ± 3	9.2 ± 1.4	9630 ±2630	11,130 ±890
B		7.36 ± 0.62	140 ± 10[c]	21.8 ± 2.6[c]	12,540 ±1600	6050 ±930[b]
A	Brain	0.750 ± 0.05	—	17.0 ± 0.9	628 ± 41	37 ± 3.0
B		0.738 ± 0.05		16.0 ± 0.5	667 ± 55	42 ± 4.0

[a] Data are 4-hr mevalonate incorporation. Each value represents an average of six, 8-month-old male rats. Results are given as mean ± SEM.

[b] Significant differences between B_{12} supplemented and B_{12} deficient groups are marked: $p < 0.05$; [c] $p < 0.01$.

tivity and sp act of cholesterol were substantially lowered in B_{12} deficient rats.

Discussion. The specific aim of these experiments described was to determine which reactions in the cholesterol biosynthetic pathway might require the presence of vitamin B_{12}, and if B_{12} involvement is confined to the liver or is a general phenomenon. It was observed that B_{12} deficiency lowered plasma total cholesterol as has been shown by Icayan and Chow (3), the component diminished being the ester fraction. Normally, 25 to 30% of plasma cholesterol is unbound and the remaining portion is in esterified form. Sperry (5) showed that there is enormous variation in total cholesterol content in plasma of healthy subjects but that the ratio of free to total cholesterol is within very narrow limits. It has been further shown by Friedman *et al.* (6, 7) that the primary regulator and endogenous source of blood cholesterol is the liver, and that hepatic disease results in an alteration in the plasma free to total ratio as demonstrated by Man *et al.* (8) and Gardner *et al.* (9). Data (Table I) demonstrated that B_{12} deficient rats had a significantly higher free to total ratio in comparison to that observed for treated rats. Since liver disease results in similar observations, it was thought that derangements in liver function might occur as an outcome of B_{12} insufficiency.

Data on total cholesterol content in liver, however, did not show consistent changes as a result of B_{12} deficiency. This observation made it highly improbable that the lower levels of cholesterol in the plasma were due to unavailability of liver cholesterol. It has been postulated by Brot *et al.* (10) that the liver synthesizes an enzyme that is released into the blood stream for plasma esterification of cholesterol. Therefore, the observation of lower cholesterol ester content in plasma of B_{12} deficient rats is suggestive of (i) lack of, or inactivation of, the liver esterifying enzyme, (ii) a faulty enzyme discharge mechanism, or possibly, (iii) a deficit in fatty acids essential for formation of cholesterol esters.

Another area considered for exploration was cholesterol formation from its precursor, acetate, the simplest metabolite participating in the biosynthesis of cholesterol and also a reactant in other metabolic processes. B_{12} deficiency resulted in increased incorporation of acetate into liver cholesterol and this enhancement was of at least 3-hr duration. Additional experimentation for which the data are not shown demonstrated that acetate pool size was unaltered in deficient animals. Other studies indicate that incorporation of mevalonate into cholesterol after 4 hr was reduced in the B_{12} deprived rats. Since evidence of Icayan and Chow (3) and our acetate data demonstrated that B_{12} resulted in an increased cholesterol synthesis from acetate, it seemed that the observed reduction of mevalonate incorporation into cholesterol was linked to the availability of this intermediate.

The testes were further selected for study because it has been shown by Lepkosky *et al.* (11) and Jones *et al.* (12) that B_{12} deficiency results in impairment of reproductive processes. Although cholesterol content in the testes of deficient rats was equal to levels observed in treated rats, radioactivity of cholesterol and per gram of tissue were significantly lowered. These observations suggest that B_{12} is involved in cholesterol biosynthesis in organs other than the liver.

Cholesterol is a precursor of adrenal steroid hormones. According to Dryden and Hartman (13), only the kidneys and pituitary deposit more B_{12} than the adrenals. The high B_{12} concentration suggests its role in adrenal metabolism. Our findings indicating a significant increase in adrenal cholesterol content and a marked drop in incorporation of mevalonate into the cholesterol of B_{12} deficient rats support this possibility. Moreover, the increased ratio of cholesterol to total lipid in B_{12} untreated animals further suggests that B_{12} deficiency may cause an alteration in the amount of some other lipid components in the adrenals.

Since cholesterol is a major constitutent of the central nervous system and B_{12} deficiency causes marked neurological changes [Holton *et al.* (14) and Holmes (15)], it therefore seemed likely that alterations in brain cholesterol content might occur. On analysis, there was no demonstrable difference in brain cholesterol content, and mevalonate incorpo-

ration into the brain cholesterol was negligible. Additional work is needed to clarify this relationship.

Summary. Cholesterol biosynthesis was studied in male rats from mothers fed a vitamin B_{12} deficient diet during pregnancy and lactation. Deficient males had significantly lower plasma cholesterol levels, the ester fraction being diminished. Adrenal cholesterol was appreciably higher, relative to levels observed in the B_{12} fed rats, while cholesterol content of testes and brain was not significantly different between the two groups. Vitamin B_{12} deficiency enhanced the rate of acetate incorporation into liver cholesterol while mevalonate incorporation remained unchanged after 30 min and 2 hr but was markedly lower in the deficient group after 4 hr. Mevalonate incorporation into adrenal and testicular cholesterol was also reduced at 4 hr but incorporation into brain cholesterol was unchanged.

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Brief Communications

Absorption of Vitamin B_{12} from the Human Colon[1]

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ABSORPTION of vitamin B_{12} from the human colon at physiological dose levels (1–5 μg) has not been observed either in the presence or absence of intrinsic factor (1, 2). Bryant and Stafford (3) showed that vitamin B_{12} is absorbed in vivo from isolated, sterilized loops of dog colon and suggested that the failure of Citrin et al. (1) to demonstrate absorption from the human colon was due to inadequacy of their antibiotic preparation.

In this investigation, the bowel of human subjects was prepared with oral kanamycin (4) and a low residue diet prior to rectal administration of physiological doses of vitamin B_{12}.

MATERIALS AND METHODS

Each of three subjects was given a nonresidue diet for 84 hr before the test dose of vitamin B_{12}. Juices only were permitted for the last 24 hr and until 10 hr after the test dose. Oral kanamycin, 1 g every 6 hr, was given from 72 to 6 hr before the test dose, and 18 hr before the dose a 1,500 ml enema of 0.9% saline was given.

The test dose of 0.8 μg ^{57}Co cyanocobalamin was dissolved in 1 ml of distilled water, made up to 250 ml with sterile Ringer solution and run at 37 C into the sigmoid colon via a catheter passed to 12 inches from the anus. In *subjects 1* and 2, 10 mg of purified hog intrinsic factor were mixed with the dose. The subjects varied their posture to facilitate movement of the dose and it was retained for at least 10 hr.

Six hours after the test dose, 1 mg of unlabeled hydroxocobalamin was given intramuscularly. Blood samples were taken at 2, 5, 8, and 10 hr after the test dose in *subject 1*; 2½, 5, and 8 hr in *subject 2*; and 8 hr in *subject 3*. Urine was collected for 48 hr and feces until no significant activity remained. Percentage of the test dose in plasma, urine, and feces was estimated as previously described (5, 6).

RESULTS

Fluoroscopic examination of *subject 1*, at the time of administration of the test dose, showed an empty colon and 1 hr later the dose, to which 15 g of $BaSO_4$ in suspension had been added, was seen to be well distributed with some of the latter in the cecum.

Radioactivity (0.18% of the dose/liter) was detected in only one plasma sample (*subject 2* at 8 hr). Radioactivity was found only in the 24- to 48-hr urine collection (0.03% of the dose) from *subject 2*. *Subjects 1*, 2, and 3 excreted, respectively, 80, 79, and 87% of the dose in the feces.

COMMENT

The colon was emptied with the nonresidue diet and enema to allow maximum contact of the test dose with the mucosa, to reduce levels of endogenous vitamin B_{12} which may have diminished absorption of labeled vitamin B_{12} (7, 8), and to reduce the substrate for intestinal bacteria. Kana-

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mycin was included to reduce the possibility of bacterial interference with absorption as may occur in jejunal diverticulosis (9).

The plasma, urine, and fecal levels of ^{57}Co vitamin B_{12} were within the range observed for pernicious anemia patients given an oral dose of ^{57}Co vitamin B_{12} (6). The results, therefore, confirm that in neither the presence nor the absence of intrinsic factor are physiological doses of vitamin B_{12} absorbed from the human colon.

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The Effect of Vitamin B_{12} on the Metabolism of Formate and Certain Formate Precursors by the Rat

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The ability of vitamin B_{12} (cobalamin) to spare the dietary requirement of animals for labile methyl donors, such as choline or methionine, is now well established (for a review see Arnstein, 1958*a*). This effect appears to be due to stimulation of synthesis of methyl groups, as shown by an increased utilization of a variety of ^{14}C-labelled precursors, including formate (Arnstein & Neuberger, 1953), methanol (Verly & Cathey, 1955), the α-carbon atom of glycine (Stekol, Weiss & Weiss, 1952; Arnstein & Neuberger, 1953) and the β-carbon atom of serine (Arnstein & Neuberger, 1953). It has also been known for some time that certain micro-organisms, notably *Escherichia coli*, require vitamin B_{12} for the biosynthesis of the methyl group of methionine (Davis & Mingioli, 1950), and considerable progress has been made towards elucidating the biochemical function of the vitamin in this reaction (Guest, 1959; Woods, 1958). There is some doubt, however, to what extent this function of vitamin B_{12} in the biosynthesis of labile methyl groups can account for its physiological importance, particularly in animal metabolism where methionine is an essential amino acid which can only reduce the requirement for vitamin B_{12} but not replace it completely.

In order to investigate this problem and the specificity of the action of vitamin B_{12} on biosynthesis of methyl groups, a comparison has now been made of the effect of vitamin B_{12} on the conversion of formate and two formate precursors, serine and histidine, into methyl groups and on the metabolism of formate and histidine into other products such as serine. Some of the results of this work have been published in a preliminary note (Arnstein, 1958*b*).

EXPERIMENTAL

Animals and diets. Albino rats of Institute stock were used, most experiments being done with litter mates (see Table 1). All animals were bred from mothers fed on a vitamin B_{12}-low diet (diet A) during pregnancy and until weaning. Deficient animals were then fed on the diets stated in Table 1, without vitamin B_{12}, and control animals were given the same diet with vitamin B_{12} at various times after weaning as described later.

Diet A had the composition (g./100 g. of diet): maize starch, 39; sucrose, 36; ethanol-extracted casein, 12; arachis oil, 6·4; cod-liver oil, 1·6; salt mixture (Glaxo Laboratories Ltd. no. DL 6), 4; L-cystine, 0·35; DL-threonine (omitted after 80 g. body wt. was reached), 0·1; succinylsulphathiazole, 0·5. Vitamins were added in the amounts (mg./kg. of diet): thiamine, 10; riboflavin, 10; pyridoxine, 10; nicotinic acid, 10; *p*-aminobenzoic acid, 10; calcium D-pantothenate, 50; inositol, 100; biotin, 0·1; pteroylglutamic acid, 2; α-tocopherol, 40; vitamin K, 1; choline chloride, 2500.

These modified diets have also been used (see Table 1): Diet A/L: identical with diet A, except that lactose replaced sucrose. Diet B: identical with diet A, except that cod-liver oil was replaced by arachis oil with vitamin A (8000 i.u./kg. of diet) and vitamin D_2 (4000 i.u./kg. of diet). Diet B/A: identical with diet B, except that succinylsulphathiazole was omitted and aureomycin (200 mg./kg. of diet) was added 3 weeks after weaning. Diet C (g./100 g.): soya-bean meal, 36; sucrose, 25; starch, 26; arachis oil, 8; salt mixture, 4; DL-methionine 0·6; succinylsulphathiazole, 0·5; vitamins as in diet B.

The extracted casein was prepared by boiling vitamin-low casein (Genatosan Ltd., Loughborough) six times with ethanol (4 vol.). This procedure decreased the vitamin B_{12} content from about 8 to less than 1 (usually 0·6) μmg./g. of protein, as estimated by microbiological assay with *Ochromonas malhamensis* and *Lactobacillus leichmannii*, kindly carried out by Dr J. E. Ford, National Institute for Research in Dairying.

Food consumption and body weight. All animals were weighed three times a week. The food consumption of deficient rats was almost the same as that of the control animals. Details of changes in body weight and of the food consumption during the experimental feeding period are given in Table 1.

Administration of labelled compounds. All labelled compounds (obtained from The Radiochemical Centre, Amersham, Bucks.) were diluted with carrier to give the following specific radioactivites, and the diluted substances were added to the diet: (*a*) Sodium [^{14}C]formate, 0·56 μc/mg. (expt. 1, 1·12 μc/10 g. of diet), 0·78 μc/mg. (expt. 2, 1·56 μc/10 g. of diet), 0·74 μc/mg. (expts. 3 and 4, 1·46 μc/10 g. of diet); (*b*) L-[2 ring-^{14}C]histidine monohydrochloride monohydrate, 0·48 μc/mg. (0·48 μc/10 g. of diet); (*c*) DL-[3-^{14}C]serine, 0·816 μc/mg. (0·816 μc/10 g. of diet). In Expt. 5, sodium [^{14}C]formate (0·73 μc/mg.) was injected intraperitoneally, 5·83 μc (8 mg.) being used for rats 63/1 and 63/2 and 2·92 μc (4 mg.) for rats 63/4 and 63/5.

Administration of vitamin B_{12}. Cyanocobalamin (Cytamen, Glaxo Laboratories Ltd., Greenford) was administered to control animals in the amounts shown in Table 1. In Expts. 1 and 2 it was given orally from weaning until the animals were killed, i.e. for 31 and 58 days respectively. In the other experiments it was injected intraperitoneally

twice weekly in equal doses, starting 17 days (Expts. 3 and 4), 12 days (Expt. 6, litter 54), 18 days (Expt. 6, litter 55) or 23 days (Expt. 7) before the end of the experiment. In Expt. 5, rat 2 was given 10 μg. on the last and fifth day and rat 5 was given 10 μg. on the last, ninth and thirteenth day before the injection of the labelled formate.

Vitamin B_{12} content of tissues. Vitamin B_{12} was estimated microbiologically with *Ochromonas malhamensis* as test organism, essentially by the method described by Ford (1953).

Isolation of metabolic products

Protein and amino acids. The animals were killed by dislocation of the spine and the viscera (liver, kidneys, intestine, spleen, heart and lungs) were quickly removed, washed with water and disintegrated in cold ethanol in an Ato-Mix blender. The tissue was extracted six times with boiling ethanol–ether (3:1, v/v) and finally with ether. The dry, defatted tissue was extracted three times with 20 vol. of aq. 10% NaCl at 100° for 24 hr. to remove nucleic acids. The residue was washed twice with water, ethanol and ether and will be referred to as protein.

The protein (approx. 4 g.) was hydrolysed in a sealed tube with 5N-HCl (10 vol.) at 105° for 24 hr. The cooled solutions were diluted with water, filtered and evaporated to dryness *in vacuo*, the last traces of HCl being removed in a vacuum desiccator over KOH. Amino acids were separated by chromatography, first on an anion-exchange resin (De-Acidite FF, The Permutit Co. Ltd., London) in the acetate form to separate the acidic amino acids, followed by a cation-exchange resin (Zeo-Karb 225, The Permutit Co. Ltd., London) for the neutral and basic amino acids, essentially as described by Hirs, Moore & Stein (1954), but with 1·5N- and 2·5N-HCl as eluent for the cation-exchange-resin column (Stein & Moore, 1949). About 100 g. of De-Acidite resin and 300 g. of Zeo-Karb 225 resin in columns of 2·5 cm. and 3 cm. diameter respectively were sufficient for the preliminary separation of the required amino acids.

Glutamic acid and aspartic acid were isolated from fractions containing the pure amino acids by evaporating the solution to dryness, removing excess of HCl in a vacuum desiccator, dissolving the residue in a little water and adding pyridine to pH 5·0. If necessary, ethanol was added to complete the crystallization. The amino acids were recrystallized from water by addition of ethanol. There was no change in their specific radioactivity on further recrystallization. Histidine was converted into the monohydrochloride monohydrate by a similar procedure.

The other amino acid hydrochlorides were converted into the free amino acids by adsorption on Zeo-Karb 215 cation-exchange resin (The Permutit Co. Ltd., London) and displacement with aq. 0·02N-NH_3 soln. The eluate was evaporated to dryness and the residue was crystallized from aq. ethanol. Serine was usually contaminated by threonine and was purified by conversion into the hydroxy-azobenzene-sulphonate, which was recrystallized two to three times from water.

In Expts. 3 and 6*b*, methionine was separated from leucine by chromatography on Whatman no. 3MM paper with pentan-1-ol–pyridine–water (7:7:6, by vol.). This procedure gave pure methionine in the first case, but in Expt. 6*b* the two samples still contained 10 and 15% of leucine respectively (estimated by quantitative paper chromatography). Since leucine was, however, non-radioactive, the specific radioactivity of the methionine (Table 3) has been corrected for this inactive impurity. In Expt. 1, methionine was oxidized to the sulphone with bromine–water and separated from leucine on a column of Dowex-50 cation-exchange resin (8% cross-linked, Microchemical Specialities Inc., Berkeley, Calif., U.S.A.) (1·5 cm. diam. × 55 cm.) with 1·5N-HCl as eluent. Methionine sulphone was eluted first. The appropriate fractions were evaporated to dryness and the free amino acid was obtained by displacement from Zeo-Karb 215 resin with dilute aq. NH_3 soln. as described above. Methionine sulphone was recrystallized from water by addition of ethanol.

The purity of all amino acids isolated was checked by paper chromatography, with phenol–water (5:2, v/v, with NH_3 in the tank) and pentan-1-ol–pyridine–water (7:7:6, by vol.).

Choline. Choline was isolated by the method of du Vigneaud, Cohn, Chandler, Schenk & Simmonds (1941). The conversion of choline reineckate into chloroplatinate was carried out either as described or, more conveniently, by the following procedure. Choline reineckate was dissolved in aq. 50% acetone and the solution was passed through a column of Zeo-Karb 225 (H^+ form) which had been washed free of excess of acid. The column was washed with aq. 50% acetone until the effluent was colourless, then with a little water. Choline was eluted with 2N-HCl and the solution was evaporated to dryness. The choline chloride was converted first into the mercurichloride complex (m.p. 170°) and finally into the chloroplatinate, as previously described (Arnstein & Neuberger, 1953). The chloroplatinate was recrystallized from water by addition of ethanol, the specific radioactivity remaining unchanged. The purity of the salt was checked by Pt analysis, the found values being within 0·4 of the theoretical value in all cases.

Choline was degraded into trimethylamine by the method of du Vigneaud *et al.* (1941), the purity of the trimethylamine chloroplatinate being checked by Pt analysis (values found were within 0·4 of the theoretical value). In Expt. 7 (deficient group), however, the sample was contaminated with ammonium chloroplatinate (see Arnstein, 1952). This sample was therefore also analysed for carbon by microanalysis and its reported specific radioactivity has been corrected for the low carbon content (Found: C, 6·87. Calc. for $C_6H_{20}N_2Cl_6Pt$: C, 13·6%).

Oxidation and excretion of formate (Expt. 5). After injection of the labelled formate (see above) the animals were placed in desiccators, through which CO_2-free air was passed slowly. The expired CO_2 was trapped in excess of 40% NaOH and converted into $BaCO_3$ by addition of $Ba(OH)_2$. The $BaCO_3$ was washed with water, ethanol and ether, dried, weighed and counted. In the experiment with the first pair of rats the NaOH was changed at 2 and 4 hr., in that with the second pair at 1, 2, 3, 4, 6, 8, 10 and 12 hr. after the injection of the formate, CO_2 being collected for a further 2 and 13 hr. respectively.

Urine was collected either separately for the first 6 hr. and the subsequent 18 hr. (rats 1 and 2) or for 24 hr. (rats 4 and 5). It was stored at −20°. Carrier sodium formate (27·5 mg. to the 6–24 hr. collection, 68·7 mg. to the other samples) was added, the formate was oxidized to CO_2 as described by Weinhouse & Friedmann (1952) and counted as $BaCO_3$.

Radioactivity measurements

^{14}C was estimated by counting 'infinitely thick' samples on 1 or 2 cm.^{2} polythene disks (Popják, 1950), with an end-window Geiger–Müller tube and Dekatron scaler. A 1 cm.^{2} disk of poly-[^{14}C]methyl methacrylate (The Radiochemical Centre, Amersham, Bucks.), specific radioactivity 1 μc/g., was used as a reference standard. The standard error of all counts is less than 5%.

RESULTS

Effect of vitamin B_{12} on the growth and food consumption of deficient rats and on the vitamin B_{12} content of their internal organs

With all casein diets there was a high mortality of some litters within a few days of birth, but survivors grew well and the administration of large

Table 1. *Food consumption, growth and liver vitamin B_{12} content of deficient and control rats*

Compositions of the diets and other experimental details are given in the text. Ages of the animals (column 4) and the initial body weights (column 7) refer to the beginning of the administration of labelled compounds, the increase being the gain in weight during the subsequent 7 days. In Expt. 5, however, the body weights are those before fasting and the animals were killed 24 hr. after injection of labelled formate.

Expt.	Litter and rat no.	Sex	Age (days)	Diet	Amount of vitamin B_{12} given	Body wt. (g.) Initial	Body wt. (g.) Increase	Food consumption (g./7 days)	Vitamin B_{12} content of liver (μmg./g.)
					[^{14}C]Formate experiments				
1	50/1	M.	50	A	1 μg./day	144	30	102·4	29
	50/8	F.	50	A	1 μg./day	114	15	80·8	67
	50/2	M.	50	A	None	146	29	106·8	7·8
	50/4	F.	50	A	None	117	10	79·3	9·1
2*a*	52/1	M.	76	A/L	2·5 μg./day	224	18	97·5	138
	52/2	M.	76	A/L	2·5 μg./day	234	12	105·0	112
	52/4	M.	76	A/L	None	217	2	99·8	9·0
	52/5	M.	76	A/L	None	230	5	104·1	10
2*b*	52/3	F.	76	A/L	2·5 μg./day	155	2	80·6	150
	52/6	F.	76	A/L	None	163	5	80·8	52*
3*a*	57/5	M.	50	C	20 μg./week	129	20	106·2	102
	57/1	F.	50	C	20 μg./week	115	8	70·5	119
	57/6	M.	50	C	None	142	23	113·2	18
	57/2	F.	50	C	None	106	14	75·7	91*
3*b*	57/7	M.	50	B	20 μg./week	178	38	116·2	102
	57/3	F.	50	B	20 μg./week	141	18	89·2	102
	57/8	M.	50	B	None	172	33	110·0	6·0
	57/4	F.	50	B	None	123	11	71·3	6·8
4	59/3	M.	47	B	20 μg./week	146	35	102·7	75
	59/4	M.	47	B	20 μg./week	142	30	94·4	58
	59/6	M.	47	B	20 μg./week	156	36	113·1	97
	59/5	M.	47	B	None	149	29	96·4	9·1
	59/7	M.	47	B	None	142	34	109·3	10·0
	59/8	M.	47	B	None	137	33	100·8	5·0
5	63/2	M.	49	B/A	20 μg.	129	—	—	45
	63/1	M.	49	B/A	None	135	—	—	8·3
	63/5	M.	57	B/A	30 μg.	176	—	—	115
	63/4	M.	57	B/A	None	176	—	—	21
					L-[2 ring-^{14}C]Histidine experiments				
6*a*	54/4	M.	48	B	20 μg./week	156	35	105·8	68
	55/2	M.	48	B	20 μg./week	138	20	81·8	105
	54/5	M.	48	B	None	158	40	114·9	12
	55/3	M.	48	B	None	160	29	99·2	18
6*b*	54/2	F.	48	B	20 μg./week	130	13	70·9	110
	54/1	F.	48	B	None	134	15	74·3	24
					DL-[3-^{14}C]Serine experiment				
7	60/3	M.	47	B	20 μg./week	142	25	110·2	125
	60/5	M.	47	B	20 μg./week	145	28	113·2	136
	60/7	M.	47	B	20 μg./week	151	30	115·4	150
	60/4	M.	47	B	None	134	14	80·7	8·8
	60/6	M.	47	B	None	145	30	108·4	2·7
	60/8	M.	47	B	None	141	31	113·1	6·8

* Unusually high values, possibly resulting from intestinal biosynthesis of vitamin B_{12}.

doses of vitamin B_{12} (1–3 μg. of cyanocobalamin/day) had little effect on growth and food consumption (Table 1). Nevertheless, the concentration of vitamin B_{12} in the liver of deficient animals was much lower than normal (Table 1), except in two cases (indicated by an asterisk) where synthesis of vitamin B_{12} by intestinal micro-organisms may have occurred. Other tissues (spleen, kidneys, heart, lung and brain) of deficient rats apparently also contained less vitamin B_{12} than those of rats fed on the same diet and given vitamin B_{12} or kept on a stock diet, but, except for brain and spleen, tissues of only one deficient animal and one animal on stock diet were examined. The values for brain and spleen (see Table 1 for details of diets and animals) were (μμg./g. of fresh tissue): deficient spleen, 5·2 (rat 63/1), 9·0 (rat 63/4), 16·1 (rat 50/3, a male litter-mate of the animals used for Expt. 1; body wt. 183 g.); normal spleen, 19·5 (rat 63/2), 55·6 (rat 63/5), 19·3 (male rat on stock diet, body wt. 178 g.); deficient brain, 2·8 (rat 63/1), 5·3 (rat 53/4), 2·6 (rat 50/3); normal brain, 13·1 (rat 63/2), 23·0 (rat 63/5); 22·3 (rat on stock diet).

Incorporation of labelled compounds into protein

The incorporation of labelled serine or histidine into the protein of mixed viscera was not increased significantly by vitamin B_{12} (Table 2). These results were confirmed by the observation that the specific radioactivity of histidine, which was isolated from the protein, was actually slightly decreased (Table 3). On the other hand, the incorporation of formate into protein was almost doubled by vitamin B_{12} (Table 2). The specific radioactivities of both serine and methionine isolated from the protein were increased to a similar

Table 2. *Incorporation of labelled serine, histidine and formate into protein*

Labelled compounds were fed for 7 days; where more than one animal was used, the average amount of isotope per rat is given. For other experimental details see text and Table 1.

Expt.	Litter and rat no.	Precursor fed: Compound	Precursor fed: μc/rat	Vitamin B_{12} given	Specific radioactivity of protein (mμc/g.)
7	60/3, 5, 7	DL-[3-^{14}C]Serine	4·58	+	40·9
	60/4, 6, 8	DL-[3-^{14}C]Serine	4·11	−	42·5
6a	54/4	L-[2 ring-^{14}C]Histidine	5·08	+	110·8
	54/5	L-[2 ring-^{14}C]Histidine	5·52	−	116·4
	55/2	L-[2 ring-^{14}C]Histidine	3·93	+	116·0
	55/3	L-[2 ring-^{14}C]Histidine	4·76	−	108·3
6b	54/2	L-[2 ring-^{14}C]Histidine	3·40	+	112·3
	54/1	L-[2 ring-^{14}C]Histidine	3·47	−	118·3
1	50/1, 8	Sodium [^{14}C]formate	10·3	+	72·0
	50/2, 4	Sodium [^{14}C]formate	10·4	−	42·4
3b	57/3, 7	Sodium [^{14}C]formate	15·0	+	51·6
	57/4, 8	Sodium [^{14}C]formate	13·4	−	26·3

Table 3. *Distribution of radioactivity in some amino acids isolated from the protein of animals fed with labelled formate or histidine*

The amino acids were isolated from the proteins of the mixed viscera (Table 2) as described in the text.

Expt.	Labelled precursor	Amino acid isolated	Specific radioactivity (μc/mole): Vitamin B_{12} present (a)	Specific radioactivity (μc/mole): Vitamin B_{12} absent (b)	Ratio of specific radioactivities (a/b)
1	Sodium [^{14}C]Formate	Serine*	76·0	42·3	1·80
		Methionine†	84·0	52·5	1·60
		Aspartic acid	1·73	1·36	1·28
		Glutamic acid	1·91	1·60	1·19
3b	Sodium [^{14}C]Formate	Serine*	62·7	23·3	2·70
		Methionine	59·0	24·7	2·40
6b	L-[2 ring-^{14}C]Histidine	Histidine‡	757	818	0·93
		Serine*	10·9	9·59	1·13
		Methionine	3·30	3·76	0·88

* Counted as hydroxyazobenzenesulphonate. † Counted as sulphone.
‡ Counted as monohydrochloride monohydrate.

Table 4. *Incorporation of sodium* [^{14}C]*formate,* DL-[3-^{14}C]*serine and* L-[2 *ring*-^{14}C]*histidine into choline and choline methyl groups by vitamin* B_{12}*-deficient and control rats*

Choline was isolated and degraded to trimethylamine as described in the text. A dash signifies that the choline sample was not degraded.

Expt.	Labelled precursor	Vitamin B_{12} given	Specific radioactivity (μmc/g.) Choline chloroplatinate	Trimethylamine chloroplatinate
1	Formate	+	401	--
		−	214	--
2*a*	Formate	+	317	--
		−	144	--
2*b*	Formate	+	384	--
		−	348	--
3*a*	Formate	+	211	--
		−	153	--
3*b*	Formate	+	412	317
		−	117	112
6*a*	Histidine	+	40·1	34·6
		−	36·6	35·6
6*b*	Histidine	+	44·4	40·8
		−	42·8	51·0
7	Serine	+	171	66·3
		−	131	21·1

Table 5. *Effect of vitamin* B_{12} *on the incorporation of sodium* [^{14}C]*formate into the choline of liver, intestine and other internal organs*

In Expt. 4, labelled formate was fed with the diet for 7 days to groups of three rats; in Expt. 5 labelled formate was injected intraperitoneally (see text and Table 1). Choline was isolated from the various tissues as described in the text.

Expt.	Tissue	Vitamin B_{12} given	Specific radioactivity of choline chloroplatinate (μmc/g.)
4	Liver	+	642
		−	307
	Intestine	+	448
		−	199
	Other internal organs (mixed)	+	284
		−	157
5	Liver	+	608
		−	292

extent, whereas the specific radioactivity of aspartic acid and glutamic acid was much less affected (Table 3).

Conversion of labelled formate, serine and histidine into choline

The conversion of formate into choline was markedly decreased by the absence of vitamin B_{12} (Table 4), provided that the animals had sufficiently low amounts of vitamin B_{12} in their livers. In Expts. 2*b* and 3*a* the difference in the specific radioactivities of the choline from the deficient and control animals was relatively small; in both cases the liver of one of the rats in the deficient group was found to contain an exceptionally high level of vitamin B_{12} (rats 52/6 and 57/2, Table 1).

The specific radioactivities of choline isolated separately from liver, intestine and the remaining internal organs were affected to about the same extent by vitamin B_{12} deficiency (Table 5) and the effect of vitamin B_{12} on the radioactivity of liver choline was the same whether the labelled formate was injected (Expt. 5) or fed with the diet (Expt. 4).

Degradation of the choline (Table 4) showed that with labelled formate or serine as precursor vitamin B_{12} increased the labelling of choline methyl groups approximately threefold. The radioactivity of the ethanolamine moiety of choline (obtained by difference between the radioactivities of choline and trimethylamine chloroplatinates) was also greatly increased by vitamin B_{12} when labelled formate was the precursor but not when labelled serine was used.

Table 4 also shows that vitamin B_{12} deficiency appeared to have little or no effect on the utilization of labelled histidine for the biosynthesis of either total choline or choline methyl groups.

The relative importance of serine, histidine and formate as precursors of choline and choline methyl groups is shown in Table 6, in which previous results have been expressed in terms of standardized radioactivities. Serine is the best precursor of choline, which is partly due to its extensive conversion into the ethanolamine moiety.

Table 6. *Effect of vitamin B_{12} on the relative utilization of serine, histidine and formate for the biosynthesis of choline*

The results given in Table 4 have been standardized for the administration of 100 μc/100 g. body wt. (average weight during the 7-day-feeding period). With serine, allowance has been made for the fact that D-serine is not converted into choline (Arnstein, 1951). The radioactivity of the ethanolamine moiety is obtained by difference and the error of the result is calculated on the assumption of 5% counting errors for choline and trimethylamine chloroplatinates. The choline of Expt. 1 was not degraded.

Expt.	Precursor	Vitamin B_{12} given	Standard specific radioactivity (μc/mole) Choline	Trimethylamine	Ethanolamine
7	L-[3-^{14}C]Serine	+	3680	1220	2460±245
		−	2990	413	2577±170
6*a*	L-[2 ring-^{14}C]Histidine	+	869	598	171±73
		−	771	572	199±67
6*b*	L-[2 ring-^{14}C]Histidine	+	1104	876	228±99
		−	1078	1003	75±104
1	Sodium [^{14}C]formate	+	1639	—	—
		−	641	—	—
3*b*	Sodium [^{14}C]formate	+	1473	969	504±121
		−	425	347	78±38

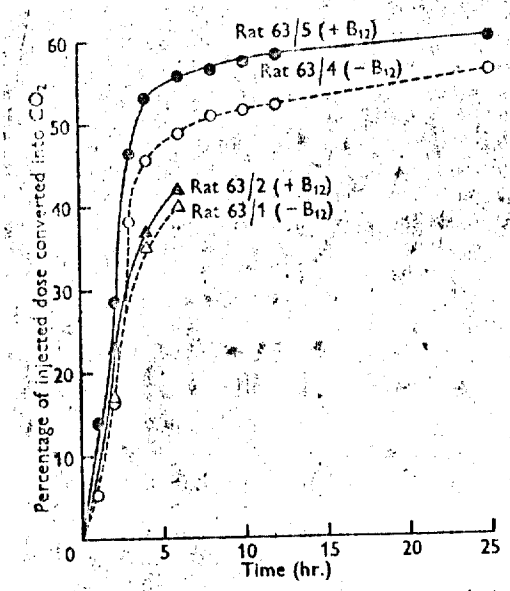

Fig. 1. Oxidation of sodium [^{14}C]formate to respiratory carbon dioxide by vitamin B_{12}-deficient and control rats. The cumulative amount of ^{14}C expired in the respiratory CO_2 during 6 and 24 hr. respectively, after injection of labelled formate, is given as the percentage of the dose. For experimental details see text.

In the presence of vitamin B_{12}, the relative efficiency of these compounds for methyl biosynthesis was serine > formate > histidine, but in vitamin B_{12}-deficient animals it was histidine > serine > formate.

Both histidine and formate are poorer precursors of the ethanolamine moiety of choline than is serine. The utilization of formate for this reaction is reduced by vitamin B_{12} deficiency, as would be expected since incorporation into serine, which is the immediate precursor of ethanolamine (Arnstein, 1951), must be an obligatory step. It should be noted, however, that the radioactivity of the ethanolamine moiety is obtained by difference and 5% errors in the counting of choline and trimethylamine could markedly affect the magnitude of the difference between the deficient and control rats. For this reason the reduction in the conversion of histidine into the ethanolamine in Expt. 6*b* may not be significant.

Oxidation and urinary excretion of formate

The effect of vitamin B_{12} deficiency on the oxidation of formate is shown in Fig. 1. The initial rate of oxidation was less in the deficient rats, but after about 2 hr. the deficient animals expired somewhat more $^{14}CO_2$ than the controls. The total oxidized in 6 or 25 hr. by the deficient animals was only slightly less than that of the controls.

The amounts of formate excreted in the urine were: rat 63/1 (deficient), 0–6 hr. 0·70 μc (12%), 6–25 hr. < 0·001 μc; rat 63/3 (control), 0–6 hr. 0·504 μc (8·6%), 6–25 hr. < 0·001 μc; rat 63/4 (deficient), 0–25 hr. 0·685 μc (23·5%); rat 63/5 (control), 0–24 hr. 0·144 μc (5·0%). Formate excretion was thus essentially complete within 6 hr. of the injection of the labelled formate and the deficient animals excreted a greater proportion of the dose than did the controls.

When these results are combined, the total amount of formate excreted and oxidized to CO_2 was: rat 63/1, 3·02 μc (51·8%); rat 63/2 (control), 2·93 μc (50·3%); rat 63/4 (deficient), 2·27 μc

(78·0%); rat 63/5 (control), 1·89 μc (65·0%). The differences between the deficient animals and the corresponding controls are thus less than 20%.

DISCUSSION

The observed stimulation of the biosynthesis of choline methyl groups from serine and formate by vitamin B_{12} is qualitatively similar to that previously obtained with rats fed on amino acid diets deficient in methionine (Arnstein & Neuberger, 1953). The greater difference in the utilization of formate by deficient and control rats in the present work is not surprising in view of the likelihood that the deficient animals were more thoroughly depleted of vitamin B_{12} since they had been specially bred from mothers also kept on a deficient diet. This interpretation is supported by the apparent correlation between the conversion of formate into choline and the vitamin B_{12} content of the liver, which must be less than about 25 μmg./g. before effects on formate metabolism can be demonstrated. The normal growth and food consumption of deficient animals suggests that the changes in formate metabolism which have been observed in this work can occur in deficiency states which are still relatively mild. These metabolic effects may thus be an early consequence of vitamin B_{12} deficiency.

Although vitamin B_{12} increased the oxidation of formate to respiratory carbon dioxide and decreased its excretion in the urine, the combined effect of these metabolic changes appears to be too small to account quantitatively for the observed increases in methyl-group biosynthesis. This conclusion is supported by the observation that vitamin B_{12} actually decreased the incorporation of formate into nucleic acid adenine and guanine (Arnstein, 1958*b*, 1959), since the radioactivity of these metabolic products should be changed similarly to that of choline if vitamin B_{12} affected mainly the oxidation and excretion of formate. It is not unlikely, moreover, that changes in the oxidation and excretion of formate could be due to a primary effect of vitamin B_{12} on formate biosynthesis from precursors such as serine, which would affect the size of the metabolic pool and hence its catabolism. That the oxidation of the hydroxymethyl group of serine to the level of formate may require vitamin B_{12} is suggested by an increased conversion of [3-^{14}C]serine into nucleic acid adenine and guanine by the vitamin (Arnstein, 1959; Arnstein & White, unpublished results). The present work shows that vitamin B_{12} also increases the reverse of this reaction, namely the reductive conversion of formate into serine. This result is noteworthy because it demonstrates that the metabolic function of vitamin B_{12} on C_1 metabolism by the rat is not restricted to methyl-group biosynthesis.

Since vitamin B_{12}, unlike folic acid, is apparently not required for the transfer of C_1 compounds at the same oxidation level, as has been shown for transmethylation (Mistry, Vadopalaito, Chang, Firth & Johnson, 1955), for the glycine–serine interconversion (Arnstein & Neuberger, 1953; Chang & Johnson, 1955; Arnstein & Stanković, 1956) and for purine synthesis from formate (Arnstein, 1958*b*), it is suggested that it functions at their interconversion by oxidation–reduction reactions. Whether the mechanism by which vitamin B_{12} stimulates the incorporation of formate into serine and methionine is similar to that involved in methionine biosynthesis from serine by *Escherichia coli* (Guest, 1959; Woods, 1958) remains to be elucidated.

It is of interest that the experiments with labelled histidine indicate that vitamin B_{12} may not be required for the utilization of this formate precursor for the biosynthesis of serine, methionine and choline. It is possible, however, that more severely deficient animals would show a requirement for these reactions. In any case, the physiological importance of vitamin B_{12} in C_1 metabolism may be due to the availability of serine, a non-essential amino acid, as a metabolic source of formate in the presence of the vitamin, whereas in its absence histidine, an essential amino acid, has to be used. One result of the relatively greater importance of histidine as a formate precursor in the vitamin B_{12}-deficient animal could be a decrease in the availability of this amino acid for other metabolic reactions, including protein synthesis.

SUMMARY

1. The metabolism of sodium [^{14}C]formate, DL-[3-^{14}C]serine and L-[2 ring-^{14}C]histidine has been studied *in vivo* in young rats depleted of vitamin B_{12} by breeding from deficient mothers which were fed on a vitamin B_{12}-low casein diet.

2. The livers of rats fed on the deficient diet contained much less vitamin B_{12} than those of control animals and other internal organs were also depleted.

3. The incorporation of serine and histidine into the mixed visceral proteins was not reduced by lack of vitamin B_{12}.

4. Administration of vitamin B_{12} to deficient rats increased the oxidation of formate to respiratory carbon dioxide and decreased the urinary excretion of formate.

5. The conversion of formate into both serine and methionine and that of serine and formate into choline methyl groups were markedly increased by vitamin B_{12}.

6. The utilization of histidine for the biosynthesis of serine, methionine and choline was not affected by the vitamin B_{12} deficiency.

7. It is suggested that vitamin B_{12} is required for the interconversion of C_1 compounds by oxidation–reduction, but not for their transfer at the same oxidation level. The possible physiological significance of this function is discussed.

I should like to thank Dr J. E. Ford for the microbiological assays of casein samples and of the livers of the first experiment and for helpful advice on the estimation of vitamin B_{12} with *Ochromonas malhamensis*. I am also grateful to Dr A. M. White for the isolation of choline in the first experiment and for advice on the use of ion-exchange chromatography in the isolation of choline and to Mrs B. Higginson for expert technical assistance throughout this work.

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The Effect of Vitamin B_{12} on the Conversion of Glycine to Choline. By H. R. V. Arnstein and A. Neuberger. (*The National Institute for Medical Research, Mill Hill, London, N.W. 7*)

In an earlier communication one of us (Arnstein, 1950) reported that methanol, formate, the β-carbon atom of L-serine, the α-carbon atom of glycine are precursors of choline methyl groups in the rat. Similar findings have recently been obtained by various American investigators. The results to be reported now are concerned with the quantitative significance of this formation of methyl groups from non-essential dietary constituents under various conditions.

Albino rats were put on the experimental diets soon after weaning. In diets A, B, C, D and E the nitrogen requirements were provided by an amino-acid mixture (18·11 % of the diet) containing the essential amino-acids with the exception of methionine which was replaced by homocysteine. The diets F and G contained 12·5 % casein. All the basal diets contained the known vitamins including folic acid (2 mg./kg. diet), with the exception of vitamin B_{12} and choline. Vitamin B_{12} concentrate (equiv. to 2·5 μg./rat/day) was added to diets A, B, D and F, choline (5 mg./rat/day) was added to diets B and C, and DL-methionine (25 mg./rat/day) was added to diets D and E. All the diets contained

2 % ^{14}C-methylene-labelled glycine (56·8 μc./mol.). After 24, 35 or 39 days the animals were killed; the two rats in each group were combined and glycine was isolated chromatographically as the crystalline dinitrophenyl derivative separately from the carcass and internal organs, whilst choline from the two sources was combined and degraded.

The two rats on diet A, which is devoid of labile methyl groups but contains vitamin B_{12}, grew at a very slow rate (0·13 g. and 0·64 g./day respectively), whilst the rats which received 5 mg. choline but no vitamin B_{12} (diet C) lost weight steadily. The rats receiving vitamin B_{12} and 5 mg. choline (diet B) grew moderately well (0·68 and 1·2 g./day respectively). The growth rate of the rats receiving the amino-acid diet and 25 mg. methionine/day was not markedly affected by the presence of vitamin B_{12}. The results of the radioactivity measurements are shown in Table 1.

tion to be made of the extent of endogenous glycine formation. The estimates from the various experiments agree and suggest that this is about 1·5–2·0 mM/100 g. body wt./day.

The activity of the ethanolamine moiety of choline is similar to that of the glycine except in the animals which had received choline in their diet. The relative activities of the two carbon atoms of ethanolamine were not determined, and the extent to which ethanolamine is derived from glycine can therefore not be accurately calculated. The activity of the trimethylamine moiety is low on diets lacking vitamin B_{12} (diet C) or where methionine has been provided (diets D and E). The activity in the methyl groups is particularly high in diet A (presence of B_{12} and absence of dietary methyl groups); the effect of vitamin B_{12} is very marked if the results for diets B and C are compared.

Recent American work (e.g. Stekol, Bennett, Weiss, Halpern & Weiss, 1950) has provided evidence on the basis of feeding experiments that vitamin B_{12} is concerned with the synthesis of methyl groups. Our present results strongly support this conclusion.

Table 1. *Specific radioactivities of substances isolated* (μc./mol.)

Diet*	Choline			Glycine	
	Total	Ethanolamine moiety	Trimethylamine moiety	(internal organs)	(carcass)
A (basal amino-acid + vitamin B_{12})	30·73	17·60	13·13	21·29	14·73
B (basal amino-acid + vitamin B_{12} + 5 mg. choline/day)	18·28	12·57	5·71	25·12	18·74
C (basal amino-acid + 5 mg. choline/day)	10·10	8·40	1·70	23·15	14·32
D (basal amino-acid + vitamin B_{12} + 25 mg. methionine/day)	19·90	15·95	3·95	24·66	15·90
E (basal amino-acid + 25 mg. methionine/day)	18·65	15·61	3·04	24·66	15·82
F (casein + vitamin B_{12})	22·87	19·63	3·24	23·16	21·27
G (casein)	29·49	23·05	6·44	24·28	21·17

* The specific activity of the glycine fed was 56·8 μc./mol.

It can be seen that the activities of the carcass and organ glycine are similar in animals which had grown moderately well, whilst in animals which had lost weight or grown poorly, the activity of the carcass glycine is much lower than that of the organ glycine even after 39 days. A comparison of the activity of the fed glycine with that isolated from the internal organs allows an approximate calcula-

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The Thyroxine-Inhibitory Action of a Series of Ethers of *n*-Alkyl 3:5-Diiodo-4-hydroxybenzoates. By J. H. Wilkinson, Mary M. Sheahan and N. F. Maclagan. (*Department of Chemical Pathology, Westminster Medical School, London*)

Following the observations that the *n*-alkyl 3:5-diiodo-4-hydroxybenzoates (Wilkinson, Sheahan & Maclagan, 1950) exhibited a definite antithyroxine action when tested by the oxygen consumption method in mice (Maclagan & Sheahan, 1950), the effect of a series of related ethers was studied. Frieden & Winzler (1949) reported the anti-thyroxine effect of the benzyl ether of 3:5-diiodo-

Pigmentation and Vitamin-B_{12} Deficiency

SIR,—Our attention has just been drawn to an excellent article published in 1944 by Dr. Bramwell Cook entitled "A Vitamin B Deficiency Syndrome Allied to Sprue"[1] in which he gives a beautiful description of the type of pigmentation described in our article of June 29 last (p. 1713). He says, "The skin overlying the knuckles of the fingers looks darker than normal, and his complexion is sallow and muddy. There may be darkness of the skin around the neck and the lips. The patient is conscious and concerned about his blackness. . . ." And again, "The common sites of pigmentation are: on the knuckles, the tongue (black spots may appear on the dorsum of the tongue), around the lips and in the nasolabial folds, on the forehead, under the eyes, on the dorsum of the ankles, back of the wrist, extensor surface of the arms, front of the neck, front of the legs below the knees."

Dr. Cook also observed that this pigmentation was associated with a macrocytic anaemia, and that both it and the anaemia responded to crude liver extract.

As far as we are aware this is the first documented description of this type of pigmentation which we now know to be associated with vitamin-B_{12} deficiency.—We are, etc.,

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VITAMIN B_{12} AND THE NUTRITIVE VALUE OF RAW SOYABEAN.

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INTRODUCTION.

OF the many leguminous seeds, soyabean occupies a unique position. In spite of the fact that soyabean contains all the essential amino acids, the protein failed to yield a biological value or growth response commensurate with its protein quality as indicated by its amino-acid composition.

The first pointer towards the explanation of this observation was provided by Osborne and Mendel (1917) who reported that isolated soyabean protein was far superior to the raw flour. Cooking the raw bean also produced an improvement by virtue of the increased palatability intake.

The improvement in the nutritive value of raw bean brought about by processing in wet heat is too well known to need enumeration (Evans and McGinnis, 1946). After the discovery of a heat labile trypsin inhibitor in soyabean (Ham and Sandstedt, 1944 ; and Bowman, 1944) animal studies on the rôle of this factor showed that it suppressed the growth of rats (Borchers *et al.*, 1948) and chicks (Ham *et al.*, 1945). Since the degree of inhibition was related to the amount of trypsin inhibitor present in the raw bean, it was proposed that the low nutritive value of raw soyabean was due to the delayed release of methionine and the improvement on heating was attributed to increased availability of methionine (Melnick, Oser and Weiss, 1946).

Another principle, toxic to growth, present in raw soyabean closely associated with the antitryptic factors but not identical with them, was demonstrated by Liener (1951) by intraperitoneal administration to rats of preparations of trypsin inhibitor from raw soyabean. Everson *et al.* (1944) and Desikachar and De (1950) showed that germination improved the nutritive value of soyabean, but this was not due to the destruction of the proteolytic inhibitors.

The factors responsible for the antitryptic activity and growth depression were isolated and studied fairly extensively (Kunitz, 1946 ; Bowman, 1946 ; Liener 1953 and 1958).

Several investigators reported their experimental observations on the beneficial effect of vitamin B_{12} on an all-vegetable ration containing particularly soyabean meal (Charkey *et al.*, 1950 ; Marfatia and Sreenivasan, 1951). These observations related to growth studies using properly heated soyabean protein. The results of feeding vitamin B_{12} on the biological value and protein efficiency ratio of raw soyabean and the *in vitro* trypsin digestion studies of raw soyabean protein and of casein with added trypsin inhibitor with and without vitamin B_{12} are reported here.

Experimental.

Biological value determinations were carried out according to the balance-sheet method as modified by Mitchell (1923-24) with rats as the test animal. The protein efficiency ratio was determined by the method of Osborne, Mendel and Ferry (1919). In both the cases the test protein was fed at 10 per cent of the diet. The endogenous urinary and metabolic fæcal excretion of nitrogen were determined by feeding a low-nitrogen diet containing 4 per cent whole egg protein, as growing rats of 60 g. to 70 g. were employed for the BV determinations. Weanling rats weighing 40 g. to 50 g. were employed for the growth studies.

The diet employed contained the following ingredients (in per cent) Osborne-Mendel salt mixture 4, groundnut oil 10, cane sugar 10, test proteins 10 and starch to 100. Vitamins were provided at the following level (in mg./kg. of diet): Thiamine 2, riboflavin 5, nicotinic acid 10, pyridoxine 2, calcium-d-pantothenate 20, and choline chloride 100. The requisite amount of vitamins was added daily to the diet cup. Vitamins A and D were provided by 'Adexolin' 2 drops per rat twice a week.

In one series, iodinated casein at 0·25 per cent level was fed for 2 weeks to deplete the rats of their vitamin B_{12} reserves (Lewis *et al.*, 1949) and these rats were employed to determine the BV of raw soyabean protein. Vitamins were fed at double the usual level during the period of feeding iodinated casein.

The influence of feeding soyabean inhibitor extract (Borchers, Ackerson and Mussehl, 1948) along with the casein diet on the BV of casein was also studied.

Vitamin B_{12} was fed to the test group at 50 μg./kg. of diet.

The excretion of amino acids during the test protein feeding was determined by circular paper chromatographic technique (Giri, Radhakrishnan and Vaidyanathan, 1952) after desalting (Baliga *et al.*, 1955).

The release of α-amino nitrogen was estimated by the method of Pope and Stevens (1938) and methionine by the method of Horn, Jones and Blum (1946). The soyabean inhibitor used in the *in vitro* studies was prepared by precipitation from an inhibitor extract (Borchers, Ackerson and Mussehl, *loc. cit.*) by adding acetone to 70 per cent concentration.

The raw soyabean flour was defatted by cold extraction using petroleum ether.

Results and discussion.

The influence of vitamin B_{12} supplementation to a diet containing raw soyabean protein is represented in the Graph.

Protein efficiency ratio.—(PER) calculated from the growth data show that vitamin B_{12} has improved the PER of raw soyabean protein from 0·35 to 0·65. The effect of vitamin B_{12} supplementation is almost similar to that brought about by feeding optimally heat processed soyabean protein.

The biological value (BV) of raw soyabean protein using normal and vitamin B_{12} depleted animals and of casein with added soyabean inhibitor are given in Table I.

GRAPH.

Growth curve of rats on raw soyabean protein diet (10 per cent protein level).

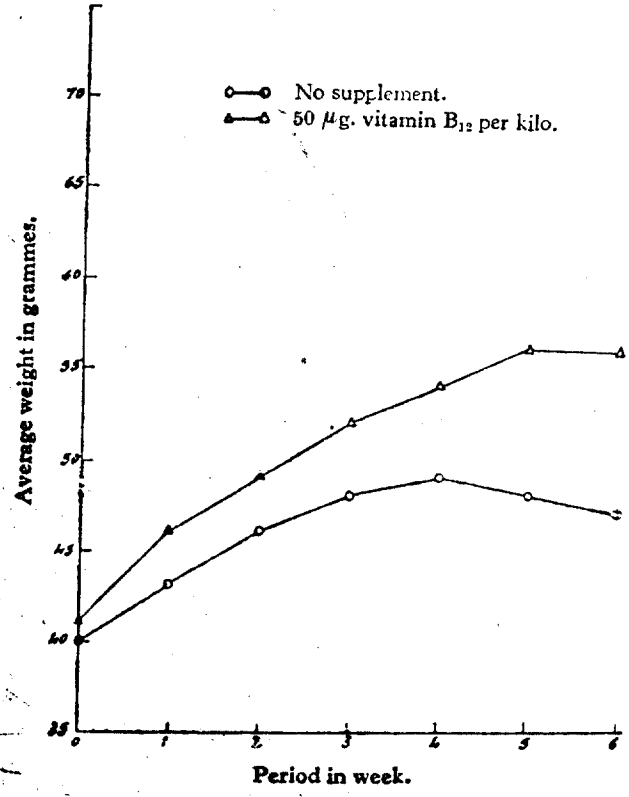

TABLE I.

Effect of vitamin B_{12} on the biological value of raw soyabean protein and casein.

Number.	Protein.	Supplement.	Nitrogen intake, mg.	Digestibility, per cent.	Biological value, per cent.
1	R.S.B.	..	420·2	82·1 ± 0·9	48·6 ± 0·4
2	R.S.B.	B_{12}	448·0	82·8 ± 1·8	77·6 ± 0·7
3	R.S.B.	Depleted	664·7	78·6 ± 1·2	66·2 ± 1·2
4	R.S.B.	Depleted + B_{12}	623·2	77·4 ± 1·2	77·4 ± 0·6
5	Casein	..	493·3	91·5 ± 1·0	86·0 ± 0·7
6	Casein	Inactive inhibitor	488·5	86·1 ± 1·4	87·4 ± 1·1
7	Casein	Active inhibitor	449·2	87·5 ± 1·8	74·7 ± 0·8
8	Casein	Active inhibitor + B_{12}	464·0	82·5 ± 1·4	85·2 ± 1·0
9	Casein	B_{12}	440·0	88·0 ± 1·1	85·7 ± 0·8

Feeding vitamin B_{12} along with a diet containing raw soyabean protein has increased the BV from 48·6 to 77·6. It is well known that the nutritive value of raw soyabean protein is low due to trypsin and growth inhibitors. Vitamin B_{12} feeding has enabled the rats to overcome the adverse effect of these inhibitors.

Vitamin B_{12} has enabled the rats, depleted of their vitamin B_{12} reserves by feeding a diet containing iodinated casein, to overcome the adverse effects of the inhibitors. The BV obtained by feeding a raw soyabean protein diet to such rats is substantially higher than that obtained on feeding the same diet to normal rats. The digestibility of the diet is significantly lower in the group of rats fed iodinated casein.

Rupp *et al.* (1951) observed that the nitrogen loss resulting from the catabolic action of thyroxine in force-fed rats on constant food intake was significantly reduced on administration of vitamin B_{12} even though the weight loss was identical. The conditions in our experiment were different in two respects, namely, the feeding was *ad lib.* and the thyrotoxic agent was not present in the feed during the period when nitrogen balance measurements were made. Ershoff (1947) reported that the beneficial effects of whole liver on growth retardation of thyroid-fed rats were correlated with increased food consumption and efficiency of food utilization. Similarly, the better utilization of nitrogen observed in the present experiment was due to increased nitrogen intake brought about by the thyroid stress during the depleting period. Another factor that should be taken into consideration would be the protective effect of soyabean meal for the immature hyperthyroid rat (Ershoff, 1949).

The experiments carried out by adding soyabean trypsin inhibitor preparation to casein support the above conclusions. The inhibitor has reduced the BV of casein from 86 to 75. Providing vitamin B_{12} along with the inhibitor has restored the BV or in other words helped the rats to overcome the deleterious effects of the inhibitors. Supplementing vitamin B_{12} alone to the casein diet does not change the BV.

The quality of the protein fed and the urinary excretion of amino acids are correlated (Pearce, Sauberlich and Baumann, 1947; Schweigert, 1947; Sauberlich, Pearce and Baumann, 1948). The urinary excretions of amino acids were followed chromatographically using butanol-acetic acid-water system. A few of the amino acids migrate as groups in this solvent, e.g. leucine and isoleucine. Since it was only intended to find out whether the urinary excretion could be correlated to the BV in the present experiment, further resolution of these amino acids that have identical Rf. values was not attempted.

TABLE II.

Effect of vitamin B_{12} on the urinary excretion of amino acids by normal and depleted rats on raw soyabean diet (mg./g. protein intake).

Number.	Amino acid.	Normal rats :		Depleted rats :	
		..	B_{12}	..	B_{12}
1	Leucines	2·1	1·7	0·8	0·6
2	Methionine and valine	2·1	1·4	0·8	0·6
3	Tyrosine	2·4	2·2	1·1	1·4
4	Alanine	2·8	2·4	0·7	1·8
5	Glutanic acid and threonine	8·2	3·4	2·5	2·6
6	Serine, glycin and aspartic acid	7·4	4·4	2·3	3·2
7	Arginine	2·5	1·6	0·7	1·1
8	Lysine and histidine	2·3	2·3	0·9	1·0
9	Cystine	2·6	1·2	..	..

In normal rats, the excretion prevailing in the group getting vitamin B_{12} supplement is lower than in the corresponding control. The excretion in the depleted group receiving vitamin B_{12} is lower than in the normal group also getting vitamin B_{12} even though the BV in both the cases are the same. In a case like this, the proportion of absorbed nitrogen excreted in urine may be equal in both the groups, but the distribution of the nitrogen into amino-acid nitrogen and other substances, such as urea nitrogen, is likely to be different. The data on hand do not allow any further interpretation.

The improvement in nutritive value brought about by supplementation of methionine to raw soyabean protein was explained by Melnick and Oser (1948) as due to the reversal of the delayed release of methionine for optimum mutual supplementation brought about by the tryptic inhibitor. Almquist and Merrit (1951) and Almquist *et al.* (1952) basing their observations on *in vitro* studies concluded that the tryptic inhibitor of raw soyabean protein reduced the tryptic digestion and release of amino acids generally, there being no evidence of a specific impairment of methionine release. Liener and Fevold (1949) also came to a similar conclusion after the results of *in vitro* digestion studies. But, in a later report, Liener, Deuel and Fevold (1949) stated that the growth-retarding action of soyabean inhibitor added to diets containing autoclaved soyabean meal was referable to interference with the availability of methionine for growth. Viswanatha and Rajagopalan (1952) from dialatometric studies of tryptic digestion of raw soyabean protein and Viswanatha Rajagopalan and De (1952) from the *in vitro* digestion studies, postulated the resynthesis of protein from the products of hydrolysis.

TABLE III.

Release of α-amino nitrogen from raw soyabean protein by trypsin with and without vitamin B_{12} (mg.).*

Time, hour.	TRICHLOROACETIC ACID FILTRATE :		ISO-ELECTRIC FILTRATE :	
	..	B_{12}	..	B_{12}
0·5	26·9	30·2	43·7	63·8
1·0	26·9	30·3	40·3	70·6
1·5	26·9	30·2	50·4	70·6
2·0	26·9	30·2	47·0	70·6
2·5	26·4	30·2	50·4	77·3
3·0	26·4	30·2	47·0	77·3
3·5	26·4	33·6	47·0	80·7
4·0	30·2	33·6	47·0	80·7
4·5	30·2	33·6	53·8	94·1
5·0	30·2	33·6	57·1	94·1
5·5	30·2	33·6	53·7	110·9
6·0	30·2	33·6	57·1	114·2
7·0	33·6	36·9	60·5	108·8
8·0	33·6	36·9	50·4	121·0
10·0	33·6	37·0	70·7	114·2
12·0	37·0	37·0	50·4	134·4
24·0	47·0	47·0	98·1	127·7

* The quantity of free amino nitrogen given, corresponds to the total amount in the substrate. Total amino nitrogen in the substrate=320 mg.

The results obtained in this experiment on the *in vitro* digestion of raw soyabean protein are in agreement with the postulate of Viswanatha, Rajagopalan and De (*loc. cit.*). During the tryptic digestion of raw soyabean protein the α-amino nitrogen in trichloracetic acid (TCA) filtrate has increased at a slower rate in the substrate enzyme system not containing vitamin B_{12}. The rise and fall of α-amino nitrogen in the case of the iso-electric filtrate may be interpreted to show that re-synthesis or aggregation takes place during the digestion. In the system containing vitamin B_{12}, the digestion is proceeding smoothly. Only in the later part, i.e. after 6 hours, is there any tendency towards resynthesis or aggregation.

The pattern of α-amino nitrogen release from casein with added soyabean trypsin inhibitor is also similar. The concentrations of α-amino nitrogen are equal at the end of 24 hours digestion in the presence or absence of vitamin B_{12} in the case of TCA filtrate, but higher in the presence of the vitamin in the iso-electric filtrate.

TABLE IV.

Release of methionine from raw soyabean protein by trypsin with and without vitamin B_{12} (mg.).*

Time, hour.	Trichloracetic acid filtrate :		Iso-electric filtrate :	
	..	B_{12}	..	B_{12}
1	1·0	1·2	1·5	2·7
2	1·0	2·8	1·7	7·1
3	1·0	4·9	1·7	15·5
4	1·2	6·9	1·7	15·5
5	1·2	8·4	2·2	28·3
6	1·2	10·2	2·2	34·7
8	1·6	10·8	2·5	35·5
10	2·2	11·4	3·2	41·1
12	2·8	12·0	3·9	42·4
24	6·1	17·9	13·2	47·8

*The quantities of methionine given, correspond to the total volume of the substrate. Total methionine in substrate = 84·4 mg.

Similarly, the release of methionine from raw soyabean protein is higher in the presence of vitamin B_{12}.

In contrast to the observations of Almquist and Meritt (*loc. cit.*), Almquist *et al.* (*loc. cit.*) and Liener and Fevold (1949), the data from the present experiment indicate that methionine is released at a comparatively faster rate than α-amino nitrogen during the tryptic digestion of raw soyabean protein in the presence of vitamin B_{12}. Re-calculating the release of α-amino nitrogen and methionine as percentage of the total at a particular time, e.g. six hours of digestion, they are 9·4, 10·5, 17·8 and 35·7 per cent of α-amino nitrogen released in the TCA filtrate and iso-electric filtrate and 1·4, 12·1, 2·6 and 41·1 per cent of methionine released.

The increase in the release of α-amino nitrogen and methionine is more in the presence of vitamin B_{12} in the case of iso-electric filtrate than in the TCA filtrate.

After 24 hours, the quantity of α-amino nitrogen released into TCA filtrate is equal in the presence or absence of vitamin B_{12} but the quantities in iso-electric filtrate are different. In the case of methionine release, the difference between the digest containing the vitamin and the digest devoid of the vitamin is persisting even though it is of a smallest magnitude. The tendency to re-synthesize is greater in the initial stages than in the later stages. The presence of vitamin B_{12} has governed the tendency of the trypsin inhibitor to tip the reaction in the forward direction with the consequence that more of α-amino nitrogen and methionine have been released.

The intermediate products of digestion of a larger size are more susceptible to re-aggregation than the smaller breakdown products as evidenced by the fluctuations in the α-amino nitrogen concentration in the iso-electric filtrate. The tendency for back synthesis is apparent between 6 and 12 hours. Even though the actual fall in α-amino nitrogen concentration is not apparent in the TCA filtrate, the rise in concentration is not smooth.

The percentage increase in release of α-amino nitrogen brought about by vitamin B_{12} during the trypsin digestion of raw soyabean protein and casein with added soyabean inhibitor are equal. This observation shows that the improvement of BV of raw soyabean protein brought about by vitamin B_{12} is solely due to the increased release of amino acids and methionine during digestion. It has, however, been assumed that the data obtained during *in vitro* digestion are not substantially different from *in vivo* digestion. On the other hand, the extent of improvement in the BV of raw soyabean protein and that of casein with added soyabean inhibitor suggest that there may be a metabolic involvement of vitamin B_{12} in the utilization of raw soyabean protein.

Apart from the two possibilities of improvement in nutritive value, namely improvement in availability and or removal of some toxic factor present in raw soyabean, the recent literature also reports that feeding raw soyabean protein diets to rats produces a hyperactive pancreas and increased secretion of enzymes in a compensatory fashion to the effects of the trypsin inhibitor (Lyman and Lepkovsky, 1957 ; Haines and Lyman, 1951). This hypertrophy of the pancreas and the drain on the endogenous amino acids brings about a state of reduced availability of amino acids for anabolism. The two postulated mechanisms lead to the same final result, namely reduced availability, the first giving reduced availability because of faulty and slow digestion and the second, due to induced wastage of assimilated amino acids. From the data available in the present experiment it is not possible to say whether hypertrophy of the pancreas was present and whether vitamin B_{12} has corrected this as well.

Summary.

1. Influence of feeeding vitamin B_{12} on the nutritive value of raw soyabean protein was determined. It was found that vitamin B_{12} increased the biological value and protein efficiency ratio to the same extent as adequate heat processing. The effect of the inhibitor and its counteraction by vitamin B_{12} was confirmed by feeding an active preparation of soyabean inhibitor along with casein.

2. *In vitro* trypsin digestion studies showed that vitamin B_{12} increased the release of α-amino nitrogen and methionine and the effect was identical when the substrate was raw soyabean protein or casein with added soyabean inhibitor.

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Influence of Vitamin B_{12} on Fecal Excretion of Cholesterol and Bile Acid of Albino rats Kept on High Cholesterol Diet

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Vitamin B_{12}, when given in high dose to rats receiving high cholesterol diet, increased the fecal excretion of bile acid without showing any appreciable increase in the excretion of cholestrol in feces. A cholesterol lowering effect in the liver and blood of these animals is also observed.

GYORGY and Rose[1] observed that vitamin B_{12} had a lipotropic effect on rats when 0·5 µg was fed daily. Ignotova[2] studied the effect of vitamin B_{12} on man and found a decreased serum cholesterol level. Hartman[3] also reported lipotropic effect of vitamin B_{12}. This method of decreasing cholesterol is of considerable interest in the study of arteriosclerosis and deserves further study. In our previous investigations[4,5], we have observed that massive dose of niacin, inositol, choline and riboflavin when given along with high cholesterol diet could lower the cholesterol contents of liver and blood. It was, therefore, thought worthwhile to investigate whether high dose of vitamin B_{12} when given along with high cholesterol diet can lower the cholesterol contents of liver and blood. Since catabolism of cholesterol consists primarily in the conversion to bile acid and appreciable amount is excreted in feces, it was also thought desirable to investigate whether or not vitamin B_{12} will influence the fecal excretion of bile acid and cholesterol of animals kept on high cholesterol diet.

Table 1 — Effect of Vitamin B_{12} on Liver and Blood Cholesterol and Fecal Excretion of Bile Acid and Cholesterol of Albino Rats Fed High Cholesterol Diet

(Number of animals used in the study are 4 and experimental period is 12 weeks. Values are mean ± SE.)

Group	Total cholesterol contents in liver, mg/100 g wet tissue	Total blood cholesterol, mg/100 ml	Daily fecal excretion of bile acids, mg/rat	Daily fecal excretion of cholesterol, mg/rat
I. Normal	253 ±10·5	42·6 ±8·2	11·8 ±1·5	8·4 ±2·7
II. High cholesterol diet	2164 ±210·5	87·6 ±12·5	33·6 ±3·1	17·8 ±5·2
III. High cholesterol diet + vitamin B_{12}	982 ±70·4	54·6 ±3·9	48·2 ±5·1	19·3 ±3·7

Albino rats weighing 120-150 g were divided into three groups. The animals of group I were given the same basal diet as was used earlier[4]. The animals of group II were kept on 1% cholesterol containing basal diet which was prepared by replacing 1 g of sucrose of basal diet by 1 g cholesterol. The animals of group III were given 1% cholesterol containing basal diet +100 µg of vitamin B_{12}/100 g of the diet. The animals were sacrificed after twelve weeks of experiment by stunning. The liver lipids were extracted with acetone-alcohol mixture (1:1). Cholesterol contents of liver, blood and feces were estimated by the method of Schoenheimer and Sperry[6]. Fecal bile acid was estimated by the method of Snell[7].

It will be evident from the data presented in Table 1 that the animals receiving high dose of vitamin B_{12} along with high cholesterol diet showed less contents of liver and blood cholesterol than animals receiving high cholesterol diet alone. The animals receiving high cholesterol diet with high dose of vitamin B_{12} also showed more excretion of bile acid in feces without any appreciable change in the fecal excretion of cholesterol. The cholesterol lowering effect of vitamin B_{12} may be attributed to increased conversion of cholesterol into bile acid which is excreted in the feces.

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α-Amylase of the Hepatopancreas of the Scorpion, *Heterometrus scaber*

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The α-amylase (α-1,4 glucan 4-glucanohydrolase, EC 3.2.1.1) of the hepatopancreas of the South Indian scorpion, *H. scaber*, has been studied. The optimum *p*H and temperature for the enzyme activity are 6·6 and 42°C respectively. Outside the *p*H range 6·0 and 8·0, and above 60°C, the enzyme is rapidly inactivated. It loses its activity on keeping at room temperature for 72 hr, while it is fairly stable at 5°C for 48 hr. Strong acid and alkali cause irreversible inactivation. The enzyme loses its activity on dialysis, but chloride ions at 0·01*M* restores the activity to 85% of the original value. Chloride ions raise the optimum *p*H of the enzyme from 6·5 to 7·5. Iodoacetate and *p*-chloromercuribenzoate have no effect indicating that —SH groups are not involved in the activity. EDTA causes inactivation, possibly due to removal of Ca^{++} ions.

THE digestive enzymes of the hepatopancreas of the scorpion, *Heterometrus scaber*, have not been investigated so far. Investigations were, therefore, undertaken on the amylotic, proteolytic and lipolytic activity of the hepatopancreas of this scorpion. The nature of α-amylase has been studied and the results are reported in this paper.

The scorpions were obtained locally and maintained in the laboratory. After the entire animal was cooled to 0°C, the hepatopancreas was removed and transferred to an ice cold mortar. It was then homogenized in a suitable buffer and the homogenate centrifuged at 0°C at 1000 rpm for 15 min. The supernatant was used as the source of enzyme. α-Amylase (α-1,4-Glucan 4-Glucanohydrolase, EC 3.2.1.1) activity was assayed according to the method of Henry and Chiamori[1], based on the estimation of the reducing sugar formed. The substrate contained 0·0067*M* NaCl, unless otherwise stated. A unit is defined as the amount which causes liberation of 1 mg of reducing substance, glucose, in 30 min under the prescribed conditions.

The α-amylase activity of the hepatopancreas of different scorpions was found to range between 24·3 and 28·3 units/g of wet tissue.

The optimum *p*H for enzyme activity is found to be 6·6. The enzyme is active between *p*H 6·0 and 8·0 while at *p*H below 6·0 and above 8·0, the activity is considerably lowered. In contrast, the human salivary α-amylase has a much wider range in that it is active between *p*H 3·8 and 9·4 with the optimum at *p*H 6·9 (ref. 2). The optimum *p*H for other mammalian α-amylases varies between *p*H 6·0 and 7·0, while it is between 5·85 and 6·0 for the α-amylase from *B. subtilis*[3], 4·8 and 5·8 for *A. orzyae*[4] and 4·75 and 5·4 for malt[5].

The optimum temperature for the enzyme activity is 42°C. The enzyme activity decreases above 42°C and the enzyme is completely inactivated above 80°C. In the human salivary α-amylase, however, the activity increases up to 40°C and decreases above this[8].

The enzyme loses most of its activity on keeping for 72 hr at room temperature, while it can be kept at 5°C for 48 hr at *p*H 6·6 without any appreciable loss of activity. At —20°C the inactivation after 72 hr is smaller than that at 5°C. The enzyme is rapidly inactivated by prior incubation at *p*H 4·0 for 1 hr while at *p*H 2·0 it is completely inactivated. At *p*H 8·0, there is only slight inactivation but at *p*H 10·0, the enzyme loses most of the activity. Thus, both strong acid and alkali cause irreversible inactivation of the enzyme.

The enzyme activity decreases to less than 10% of the original value on dialysis for 48 hr at 5°C against NH_4OH (*p*H 7·5) and using dialysed substrate (dialysed for 48 hr against 0·1*M* phosphate buffer, *p*H 6·6) for enzyme assay. The activity is restored almost to its original value by 0·01*M* NaCl. Other anions, viz. Br^-, I^-, NO_3^- have a similar effect, but to a smaller extent. Br^- restores the activity to 83% of the original value, while NO_3^- and I^- restores the activity to 58 and 37% respectively. No activation has been obtained with SO_4^{2-}, $OOCCH_3^-$ or CNS^-. The activation by Cl^- is independent of the cation present. KCl, CaCl, and $MgCl_2$ have more or less the same effect as NaCl. This effect of anions is similar to that reported for mammalian α-amylases, particularly the requirement of Cl^- ions.

The activation of the dialysed enzyme by chloride ions depends on the concentration of Cl^- ions, increasing from 0·001*M* (activity restored to 50% of the original value) to a maximum at 0·01*M* (activity restored to 95% of the original value). Increasing the chloride ion concentration above this has no further activation effect.

Activation of dialysed enzyme by Cl^- ions has been found to be a function of *p*H[6]. In the absence of Cl^- ions, the optimum *p*H for enzyme activity using dialysed enzyme is 6·5 while in the presence of chloride there is a shift of optimum *p*H to 7·5. Similar shift of the optimum *p*H towards alkaline side by chloride ions has been reported for human salivary α-amylase[7]. It is suggested that this may be due to the formation of an enzyme chloride complex and shifting of the *p*H curve to the alkaline side.

Iodoacetate and *p*-chloromercuribenzoate (at a concentration of 1 mmole) have no effect on enzyme activity. This is in accordance with the results

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Vitamin B_{12} content of some articles of Indian diets and effect of cooking on it

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Vitamin B_{12} deficiency due to dietary inadequacy is not uncommon in India (Banerjee, Hosain & Chatterjea, 1959; Banerjee & Chatterjea, 1960). To know how to prevent this deficiency, it is necessary to have precise information on the distribution of vitamin B_{12} in Indian foods and the availability of the vitamin after cooking. Our study was undertaken to provide this information.

EXPERIMENTAL

The substances analysed were muscles from fourteen varieties of fish and prawn, muscle and liver of the goat, duck egg (yolk), cow's milk and twenty items of vegetable origin, including rice and pulses. Materials were analysed in (*a*) the natural raw (uncooked) state, (*b*) after boiling, (*c*) after cooking (frying and boiling).

Raw material. The fresh material (5 g) was homogenized with distilled water and transferred into a flask. To this homogenate, 25 mg of powdered papain (Biddle Sawyer & Co. Ind. Pvt. Ltd) and 0·1 ml of 1% (w/v) sodium cyanide solution were added, and the mixture was incubated under toluene at 37° for 72 h. For vegetables, 10 mg papain were used. After incubation, 40 ml of acetate buffer (pH 4·5) were added to the homogenate, which was then autoclaved at 10 lb pressure for 15 min and cooled, and the volume was made up to 200 ml. Next the extract was filtered and from the clear filtrate a measured portion was analysed for its vitamin B_{12} content.

Boiled material. The fresh material (5 g) was boiled with distilled water for 15 min and homogenized. The homogenate, together with the boiling water, was transferred to a flask and was treated similarly to that from the raw material.

Cooked material. The fresh material (5 g) was fried with mustard oil in an open aluminium pan for 10 min at a temperature varying between 110° and 120°. The fried material was then boiled with distilled water for 15 min and homogenized. The homogenate, together with the cooking water, was transferred to a flask and treated in the same way as the raw materials.

Control. The requisite amount of powdered papain and 0·1 ml of 1% (w/v) sodium cyanide solution were added to 30 ml distilled water and incubated under toluene at 37° for 72 h. After incubation, the mixture was treated in the same way as the raw material and its vitamin B_{12} activity was measured.

Vitamin B_{12} assay. Vitamin B_{12} was assayed by the method of Ross (1952) with *Euglena gracilis* var. *bacillaris* as test organism.

RESULTS

Vitamin B_{12} in the raw materials. Articles of vegetable origin did not show any vitamin B_{12} activity. Results obtained with those of animal origin in the raw state, after boiling or after cooking, are recorded in Table 1. Results shown in the table are the means of three observations for each sample. Some of the materials were analysed on two different occasions and are represented by two mean values.

Goat liver was found to be the richest source of vitamin B_{12} examined. A fair amount was present in nine out of fourteen varieties of fish, of which Tapsey (*Polynemus paradiseus*) and Tangra (*Mystus vittatus*) seemed to be the richest. Goat muscle was found not to be a good source of the vitamin. Egg yolk was a better source than goat muscle or some fish.

Table 1. *Vitamin B_{12} activity* (Euglena gracilis *assay*) *of articles of Indian diets, and effect of boiling and cooking*

Item	Local name	Vitamin B_{12} activity (μg/100 g fresh weight) Raw	Boiled	Cooked	Loss after cooking as a percentage of activity of boiled material
Fish (muscle):					
Catla buchanani	Catla	0·7	1·6	0·48	70·0
		2·08	4·5	0·61	86·4
Lates calcarifer	Vetki	3·4	1·0	0·5	50·0
Labeo rohita	Rui	3·6	—	—	—
		6·7	1·3	0·08	93·8
Mystus vittatus	Tangra	1·18	3·08	1·0	67·5
		7·5	4·85	3·7	23·7
Heteropneustis fossilis	Singee	4·04	1·36	0·8	41·1
Clarias batrachus	Magur	3·7	2·9	0·3	89·6
Anabas testudineus	Kai	1·9	1·1	0·65	40·9
Mugil parsia	Parsey	3·6	1·7	1·1	35·2
		5·6	2·7	2·0	25·9
Glossogobius giuris	Bele	2·2	—	2·2	—
Ophiocephalus striatus	Shole	1·0	—	0·3	—
Apocryptes lanceolatus	Guley	2·6	—	0·24	—
Wallago attu	Boal	4·6	1·36	1·32	—
Hilsa ilisha	Ilish	4·0	4·5	0·16	96·4
Polynemus paradiseus	Tapsey	8·0	1·0	0·47	53·0
Prawn	Bagda, chingree	1·01	—	0·45	—
Meat:					
goat muscle	Mangsha	1·0	0·26	0·08	69·6
		1·26	—	—	—
goat liver	Mete	40·0	104	26·0	75·0
		100	—	—	—
egg, duck	Dim (patihans)	—	6·0	—	—
Milk, cow's	—	—	4·8*	—	—

* μg/l.

Vitamin B_{12} in the boiled materials. The effect of boiling was studied in eleven varieties of fish; in nine, the values after boiling were lower than in the raw materials. In goat muscle also the value was lower after boiling. In three varieties of fish, and in goat liver on one occasion vitamin B_{12} activity was higher after boiling than in the raw

state. Higher values were noted on two occasions in a single variety of fish (*Catla buchanani*) and on one occasion in the other two types (*Mystus vittatus* and *Hilsa ilisha*). Egg yolk and cow's milk were found to contain significant amounts of the vitamin after boiling.

Vitamin B_{12} in the cooked materials. Except for one variety of fish (*Glossogobius giuris*), the vitamin B_{12} activity of the materials was lower after cooking than when they were either raw or boiled. This low value was noted even for the four articles that showed higher values after boiling. Vitamin B_{12} activity after cooking was less than that after boiling in twelve out of thirteen materials studied. The amount lost in cooking as a percentage of that left after boiling varied from 23·7 to 96·4.

DISCUSSION

The figures for the vitamin B_{12} contents of Indian fish agreed well with the results recorded by Teeri, Loughlin & Josselyn (1957) for some of the species of fish consumed in New England, USA. Our values also compare well with those recorded by Braekkan (1959) and Barrett & Widdowson (1960). But the values reported by Sreenivasamurthy, Swaminathan & Subrahmanyan (1955), Ueno (1956) and Love (1961) were somewhat higher. The vitamin B_{12} content of goat muscle was low and similar to those reported for mutton and lamb leg (Schweigert, Scheid & Marquette, 1951; Barrett & Widdowson, 1960). Goat liver was found to be the richest source of vitamin B_{12} examined, the value found being similar to that given by Shenoy & Ramasarma (1954) and also to that for beef liver reported by Schweigert *et al.* (1951). Our value for goat liver was much higher than that recorded by Sreenivasamurthy *et al.* (1955). A low value was also recorded for sheep liver (Barrett & Widdowson, 1960). Egg yolk contained a significant amount of vitamin B_{12}, similar to that reported by Sreenivasamurthy *et al.* (1955). A lower value for hen's egg was reported by Barrett & Widdowson (1960).

The vitamin B_{12} content of cow's milk was found to be close to that reported by other workers (Collins, Harper, Schreiber & Elvehjem, 1951; Antener, 1958–9; Sreenivasamurthy, Nambudripad & Iya, 1953; Macy, Kelly & Sloan, 1953). The vitamin B_{12} content of raw whole milk, determined by rat assay, ranged between 5·5 and 9·4 μg/l. (Hartman, Dryden & Riedel, 1956). Gregory, Ford & Kon (1958) reported wide variations in the vitamin B_{12} content of cow's milk. The vitamin B_{12} activity of goat liver, egg yolk and fish agreed well with the values for lamb liver, egg yolk and fish, respectively, as reported by Lichtenstein, Beloian & Murphy (1961).

A comparative analysis of the vitamin B_{12} contents of the raw and boiled materials would indicate that boiling as done in our experiments may lead to a considerable loss of the vitamin. This observation cannot be satisfactorily explained, since cyanocobalamin itself was not destroyed. The explanation may lie in the method of preparing the samples for assay. To begin with, the raw food was homogenized and incubated, without prior heat treatment, under toluene for 72 h at 37° in presence of papain. Toluene did not necessarily prevent all bacteria from growing, and there was the possibility that bacterial synthesis of vitamin B_{12} had occurred during this incubation

period. Further, the natural enzymes present in the raw food might have been active. In contrast, in the heated foods there was less likelihood of bacterial growth occurring, and the natural enzymes had been inactivated. These two factors could result in a higher vitamin B_{12} activity being measured in the raw foods, either because the natural enzymes were more effective in releasing the vitamin than papain or because bacterial synthesis of vitamin B_{12} had occurred.

Increase in vitamin B_{12} activity, as found in three varieties of fish and goat liver, could be due to better extraction of the vitamin by boiling.

Reports on the effect of cooking are scanty. We found that the vitamin B_{12} activity of almost all our materials was much lower in the cooked state than in the boiled state. Thus cooking entailed considerable loss in activity. In comparing the activities of the boiled and the cooked material, there was hardly any possibility that the results had been influenced by the variable factors of enzymic release or bacterial synthesis or both.

SUMMARY

1. To ascertain the distribution of vitamin B_{12} in Indian diets, several varieties of fish, goat muscle and liver, egg yolk and cow's milk were assayed. The effect of boiling and cooking on the availability of the vitamin was also investigated.

2. The material was homogenized in the natural raw state, or after boiling for 15 min or after cooking (frying and boiling). All three homogenates were digested with papain and sodium cyanide and extracted with acetate buffer. The vitamin B_{12} activity of the extracts was determined microbiologically with *Euglena gracilis*.

3. The largest amount of vitamin B_{12} activity was found in goat liver; most of the fish contained a fair amount.

4. Considerable amounts of vitamin B_{12} activity were destroyed by cooking. The loss, as a percentage of the activity after boiling, varied from 23·7 to 89·6%.

5. The results of the study are discussed in relation to other published work.

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*A COENZYME CONTAINING PSEUDOVITAMIN B_{12}**

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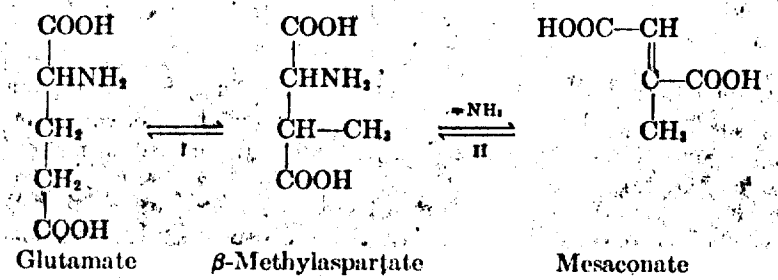
Vitamins of the B_{12} (cyanocobalamin) group apparently participate in several metabolic processes.[1] For example, Helleiner and Woods[2] reported that a cell-free extract of an *Escherichia coli* mutant, which requires either methionine or vitamin B_{12} for growth, forms methionine more rapidly in a reaction mixture containing homocysteine, serine, and adenosine triphosphate when supplemented with vitamin B_{12}. This indicates that the vitamin participates at some point in methyl group formation or transfer. More recently, Wagle *et al.*[3] found that vitamin B_{12} stimulates incorporation of amino acids into the proteins of a microsome-soluble protein system of vitamin B_{12}-deficient rats. Inhibition experiments with an unspecified anti-B_{12} compound indicated that the vitamin is involved in the activation of amino acids. Evidence for the involvement of vitamin B_{12} in the synthesis of deoxyribose by *Lactobacillus leichmanii*[4, 5] and in the reduction of dithio groups[6, 7] has also been presented.

Although these and other studies[1] have identified several metabolic processes in which the cobalamins participate directly or indirectly, the precise roles of these compounds have not been established. No specific enzymatic function has been demonstrated, and no coenzyme form of the vitamin has been described.

We wish to report the isolation of a coenzyme form of pseudovitamin B_{12} which is required for the decomposition of glutamate by particle-free extracts of *Clostridium tetanomorphum*. The coenzyme is involved in the rearrangement of the carbon skeleton of glutamate to form a branched chain amino acid, β-methylaspartate.

Previous studies in this laboratory[8] have shown that the fermentation of glutamate by *C. tetanomorphum* occurs by a sequence of reactions that is entirely different from the tricarboxylic acid cycle. A key compound in this sequence is mesaconate, a branched-chain C_5 dicarboxylic acid. The reversible conversion of glutamate to mesaconate and ammonia involves the following two reactions:



Glutamate β-Methylaspartate Mesaconate

Reaction I has been shown to require a coenzyme that can be removed from crude extracts by treatment with charcoal.[8a] Such extracts can be reactivated by the addition of boiled extract or purified coenzyme (Table 1). Estimation of the rate of reaction I and of coenzyme concentration is based upon the rate of mesacon-

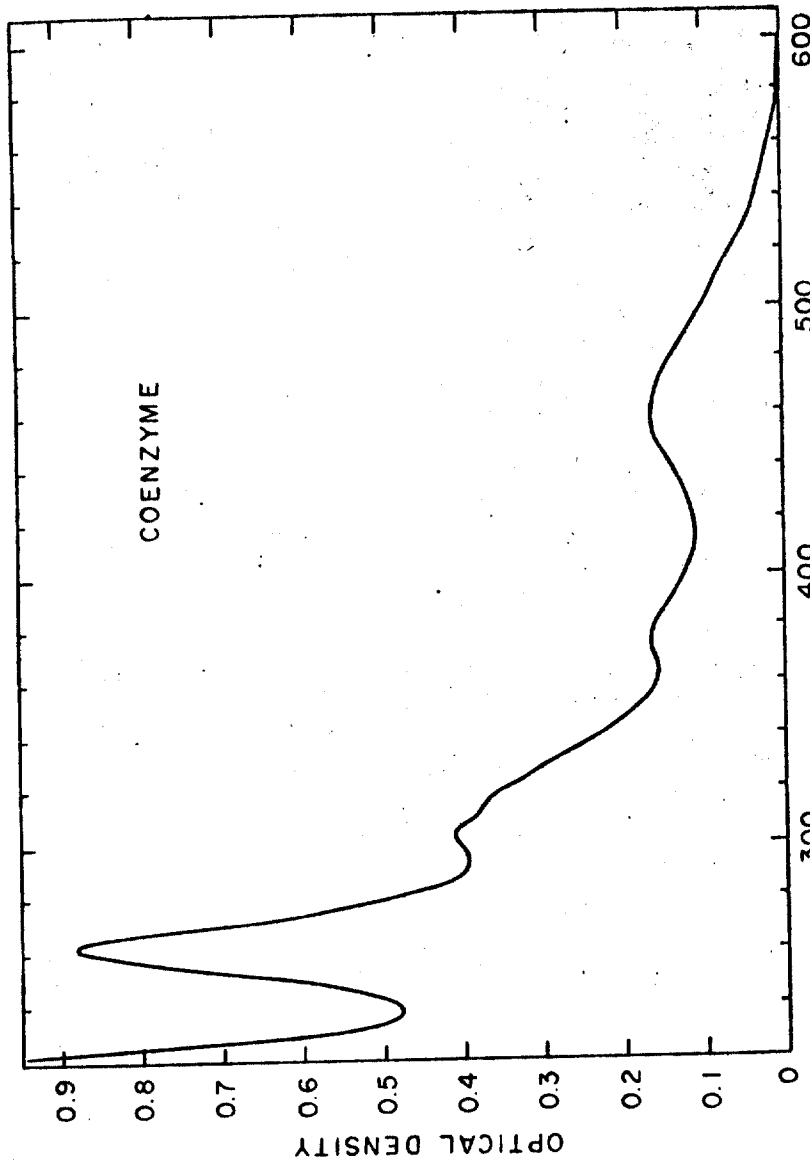


FIG. 1.—Absorption spectrum of the active coenzyme, 1.8×10^{-5} *M*, in 0.03 *M* sodium acetate pH 6.7.

ate formation, determined spectrophotometrically at 240 mμ, in a system containing an excess of β-methylaspartase,[9] which catalyzes reaction II. For coenzyme assay, a charcoal- and protamine-treated extract, containing the apoenzyme for reaction I, is also added in excess.

We have developed a method for the purification of the coenzyme in micromolar amounts from *C. tetanomorphum* by the use of columns of alumina, Dowex-1, and Dowex-50. Fractions containing the most highly purified coenzyme are yellow-orange in color and show a distinctive absorption spectrum (Fig. 1), with maxima

TABLE 1*

COENZYME REQUIREMENT FOR CONVERSION OF GLUTAMATE TO MESACONATE

Expt.	Conditions	ΔO.D. 240 mμ/min
I	Crude extract, 0.2 ml.	0.180
	Charcoal-treated extract, 0.2 ml.	0.008
	Charcoal-treated extract, 0.2 ml. + heated extract, 0.2 ml.	0.142
II	Enzyme	0.002
	Enzyme + 0.02 ml. coenzyme solution	0.035
	Enzyme + 0.04 ml. coenzyme solution	0.076
	Enzyme + 0.08 ml. coenzyme solution	0.118

* All reaction mixtures contained Tris-chloride buffer pH 8.2 0.05 *M*, KCl 0.01 *M*, $MgCl_2$ 0.001 *M*, and potassium L-glutamate 0.01 *M* in a total volume of 1 ml. In Expt. I the crude extract contained 30 mg. protein per ml., and the charcoal-treated extract contained 28 mg. protein per ml.; the heated extract was prepared from the crude extract by heating for 10 minutes at 90° C. In Expt. II the enzyme was 0.05 ml. of a charcoal- and protamine-treated extract containing 23 mg. protein per ml.; the purified coenzyme solution was 9.1×10^{-6} *M* based on the assumption that the extinction coefficient in 0.1 *M* KCN at 367 mμ is the same as for vitamin B_{12}.

at 263, 303, 374, and 457 mμ. When a solution of the coenzyme is exposed to light from a tungsten lamp, the color changes gradually from yellow-orange to red, and the absorption spectrum is modified to give maxima at 261, 351, 407, 495, and 525 mμ (Fig. 2). The latter spectrum is similar to that of vitamin B_{12} and related compounds.[10] The change in the spectrum accompanies inactivation of the co-

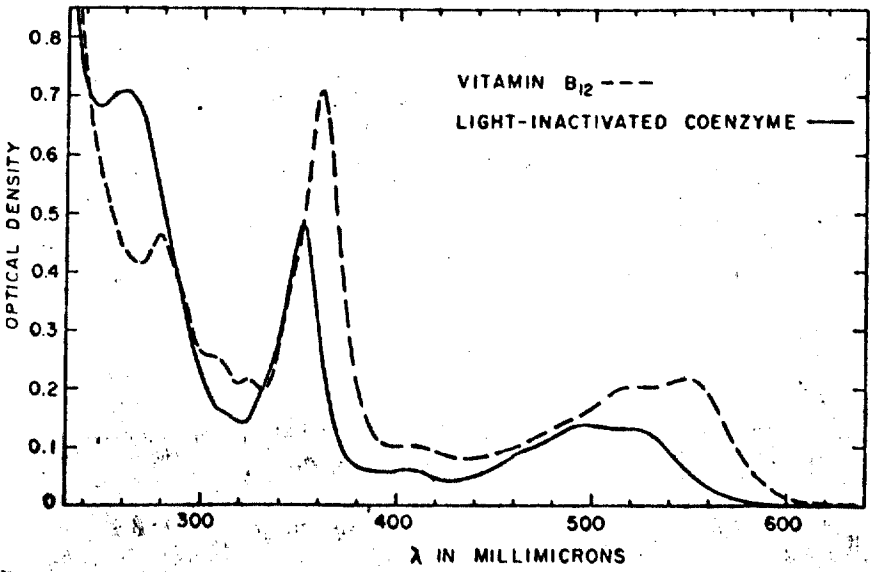


FIG. 2.—Absorption spectra of 2.6×10^{-5} *M* vitamin B_{12} and 1.8×10^{-5} *M* coenzyme inactivated by exposure to a 100-watt tungsten lamp for 105 minutes.

enzyme (see below). When 0.1 *M* KCN is added to the coenzyme, the color changes to reddish-purple, and the spectrum is characteristic of the dicyanide form[10] of the vitamin B_{12} group (Fig. 3). Further evidence for a structural similarity with this group is provided by the demonstration that the coenzyme contains approximately equimolar amounts of cobalt[11] and cyanide. Adenine, ribose, ammonia, and

phosphate are released from the coenzyme by acid hydrolysis, indicating that it is a derivative of pseudovitamin B_{12}.[12] A conspicuous difference between the spectra of the vitamin B_{12} group and the coenzyme is the relatively higher absorption of the latter in the 263–276 mμ region. This indicates that the coenzyme contains one or more additional light-absorbing groups. In confirmation of this conclusion we have

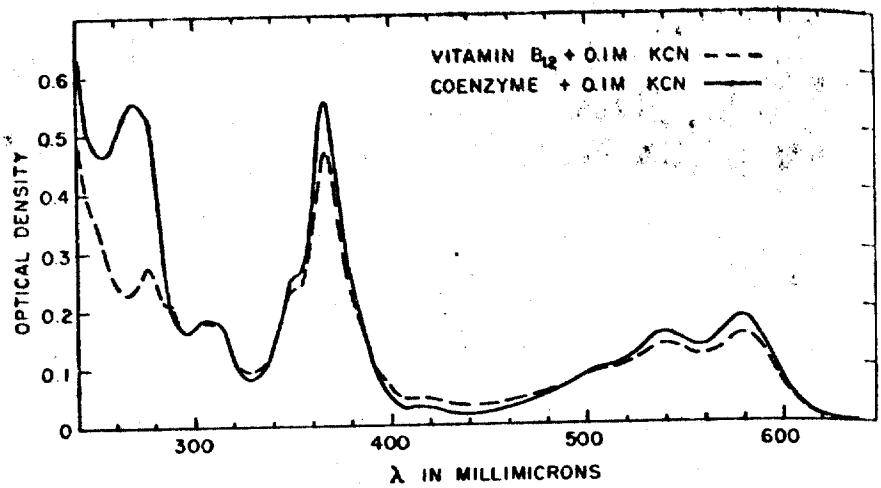


FIG. 3.—Absorption spectra of 1.5×10^{-5} *M* vitamin B_{12} and 1.8×10^{-5} *M* coenzyme in 0.1 *M* KCN.

observed that exposure of the coenzyme to light or to mild acid hydrolysis splits off a colorless compound that contains adenine or a closely related substance.

The connection between the yellow-orange compound (Fig. 1) and coenzyme activity was established in several ways. Throughout purification of the coenzyme, the activity was observed always to accompany the yellow-orange color. Quantitative enzymatic assays on highly purified column peak fractions showed a close proportionality between activity and optical density at 263, 303, and 457 mμ. Further evidence was provided by an experiment in which the change in absorption spectrum, on exposure of the coenzyme to light for various intervals, was correlated with enzymatic ac-

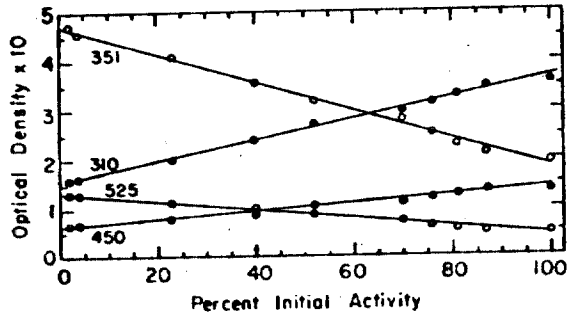


FIG. 4.—Proportionality between enzymatic activity and spectral changes during inactivation of the coenzyme by visible light. A 1.8×10^{-5} *M* solution of purified coenzyme in 0.03 *M* sodium acetate pH 6.8 was exposed at 0° C. to a 100-watt tungsten lamp at a distance of 6–30 cm. At intervals during 105 minutes, samples were removed for determining coenzyme activity, and the spectrum by means of a Cary spectrophotometer. The indicated wave lengths are expressed in millimicrons.

tivity. The decline in activity was found to be directly proportional to the change in optical density at several different wave lengths (Fig. 4). These observations indicate that coenzyme activity and the spectrum belong to the same compound. Neither vitamin B_{12} nor pseudovitamin B_{12}[12] is active in the enzymatic reactions.

We conclude that the coenzyme is a derivative of pseudovitamin B_{12} containing one or more additional purine moieties. The light-sensitivity of the coenzyme and the large shift of the peak in the visible region to longer wave lengths on exposure to light suggest that a functionally important substituent group is attached both to cobalt and to some part of the conjugated double-bond system of the porphyrin-like structure.

The precise role of the coenzyme in the interconversion of glutamate and β-methylaspartate is not yet known.

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*ON THE REGULATION OF FATTY ACID BIOSYNTHESIS BY LIPOGENIN**

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We have shown[1] that a substance could be extracted from liver or yeast which, in catalytic amounts, markedly increased the rate of fatty acid synthesis in cell-free preparations from fasted rats. We now propose the name of "lipogenin" for this substance. The lipogenin used in these experiments was prepared from yeast

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Isolation and Properties of Crystalline Cobamide Coenzymes Containing Benzimidazole or 5,6-Dimethylbenzimidazole*

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The isolation of three cobamide coenzymes has been briefly reported (1, 2). Methods used in the assay and purification of the adenylcobamide coenzyme have been described in some detail (3). This paper describes methods used in the isolation of the benzimidazolylcobamide and 5,6-dimethylbenzimidazolylcobamide coenzymes, both of which have been obtained as crystalline products. Some properties of the coenzymes are also reported.

EXPERIMENTAL

Materials and Methods

Bacteria—Clostridium tetanomorphum strain H1 and *Propionibacterium shermanii* ATCC 9614 were used as sources of the coenzymes. *C. tetanomorphum* was grown in a glutamate-glucose-yeast extract medium (3), modified by the inclusion of 10^{-4} M benzimidazole or its 5,6-dimethyl derivative as required. *P. shermanii* was grown under anaerobic conditions in a medium containing glucose, cornsteep liquor, cobaltous nitrate, and buffer.[1] To avoid excessive acidification of the medium during fermentation, the culture was stirred continuously and was neutralized at least twice daily to pH 7.6 with 2 N NaOH. Cultures were incubated for 4 to 6 days at 30° before harvesting the cells with a Sharples centrifuge. The cell paste was frozen as soon as it was removed from the centrifuge and was stored at −10° until used.

Chemical and Physical Methods—Most of the methods used in this investigation have already been reported (3). Additional methods are described or referred to in appropriate sections of the text.

Coenzyme Activity Assay—The activity of cobamide coenzyme preparations was estimated by the spectrophotometric assay, based on the catalytic function of the coenzymes in the enzymatic conversion of glutamate to mesaconate (3). Activity measurements can be used to determine coenzyme concentration by direct comparison with the activity of a sample of purified coenzyme of known concentration. Without such a standard only relative coenzyme concentration can be determined. The molar activities of the BC[2] and DBC coenzymes are approximately 5 and 0.1 times as great, respectively, as that of the AC coenzyme (see Table VI). These relative molar activities are applicable only when activity comparisons are made with data obtained from the linear portion of the rate-coenzyme concentration curves.

Estimation of Coenzyme Concentration—The concentration of purified coenzyme preparations, containing only a single colored component, was estimated by absorbancy measurements at 519 mμ or 261 mμ for the BC coenzyme and at 522 mμ or 260 mμ for the DBC coenzyme in 0.01 M potassium phosphate buffer, pH 6.7 to 6.8. The corresponding molar extinction coefficients are given in Table V.

Isolation of Coenzymes

The BC and DBC coenzymes were isolated by small modifications of the methods used to obtain the AC coenzyme (3). The former coenzymes are slightly less basic than the AC coenzyme and therefore are eluted from a Dowex 50, pH 3 column somewhat more rapidly by a sodium acetate buffer between pH 6 and 7. Also, the BC and DBC coenzymes, unlike the AC coenzyme, crystallize readily from water or water-acetone mixtures.

All operations were carried out in the dark or in very dim light. The temperature of solutions was kept at 0–4°, except that phenol extractions were done at room temperature, 15–23°.

BC Coenzyme—As starting material, 3.86 kg of frozen cells of *C. tetanomorphum*, grown in the presence of benzimidazole, were used.

The first four steps (ethanol extraction, treatment with Dowex 50-Na^+, treatment with Dowex 2-OH^-, and phenol extraction and displacement back into water) were done as previously described (3). The resulting aqueous solution contained 59 μmoles of coenzyme in 70 ml. The solution was acidified to pH 3.2 with 7 ml of 1 N HCl and was then diluted to 400 ml to reduce the salt concentration.

The acidified coenzyme solution was passed into a 1 cm diameter × 80 cm high column of Dowex 50, pH 3, 2% cross linked, 200 to 400 mesh, prepared as previously described (3). After collection of the "pass through" solution (Fractions 1 to 37), the column was eluted first with 0.03 M sodium acetate, pH 3.2

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[1] *P. shermanii* was grown in a commercial laboratory and consequently the exact composition of the medium is not available. The culture conditions were generally similar to those described by Leviton and Hargrove (4) and by Hinz (5) for the synthesis of vitamin B_{12} by propionic acid bacteria.

[2] The abbreviations used are: BC, benzimidazolylcobamide; DBC, 5,6-dimethylbenzimidazolylcobamide; AC, adenylcobamide.

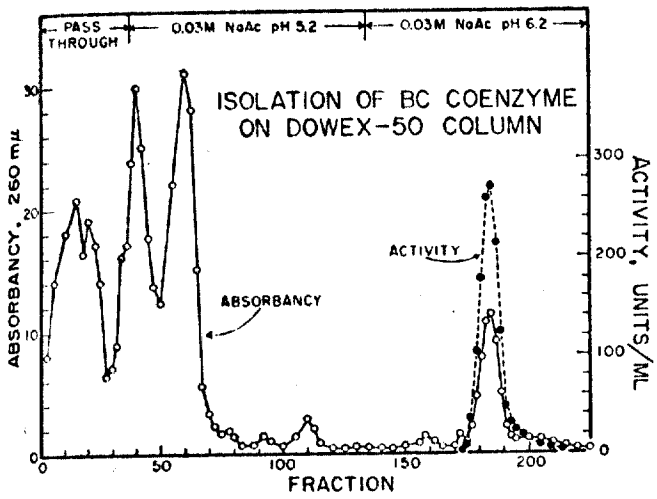

FIG. 1. Isolation of BC coenzyme on a Dowex 50W column

(Fractions 38 to 134), and then with 0.03 M sodium acetate, pH 6.2 (Fractions 135 to 240). The volume of each fraction was about 16 ml. The coenzyme eluted in a well isolated and rather symmetrical 260 mμ absorbancy peak located between Fractions 175 and 195 (Fig. 1). The peak fractions were reddish orange in color.

Coenzyme activities of alternate peak fractions were determined and their apparent specific activities were calculated. The results (Table I) show that the fractions containing relatively high concentrations of cobamide had fairly constant apparent specific activities, indicating that they were relatively homogeneous with respect to light-absorbing components.

Fractions 178 through 193, containing an estimated 53 μmoles of BC coenzyme in 284 ml, were combined and the coenzyme was extracted into phenol and displaced back into water by the addition of ether-acetone (3). The resulting solution, containing 48 μmoles of coenzyme in a volume of 8 ml, was placed in a

TABLE I

Properties of benzimidazolylcobamide coenzyme peak fractions from Dowex 50, pH 8 column

Fraction	Total cobamide*	Apparent specific activity†
	μmoles/ml	*activity units/μmole*
173	0.049	60
175	0.027	390
177	0.061	670
179	0.131	870
181	0.212	920
183	0.298	970
185	0.314	960
187	0.256	980
189	0.138	1000
191	0.062	820
193	0.040	870
195	0.033	800
197	0.032	650
201	0.036	470
205	0.033	270

* Calculated from the absorbancy at 261 mμ, assuming a molar extinction coefficient of 34.7×10^6 cm² per mole.

† Estimated from the spectrophotometric coenzyme activity assay and the cobamide concentration given in *Column 2*. The activity unit represents a change of 1 absorbancy unit per minute at 240 mμ under the conditions of the assay (3).

vacuum desiccator over concentrated sulfuric acid and left at 4°. After 2 days the volume had been reduced to about 4 ml and the walls and bottom of the container were found to be incrusted with small rectangular or diamond-shaped crystals which were either red or yellow in color depending upon the angle of view (Fig. 2*A*). The solution was left under reduced pressure for another day during which time it concentrated to approximately 2.5 ml and additional crystalline material separated. The mother liquor was removed and the crystals were

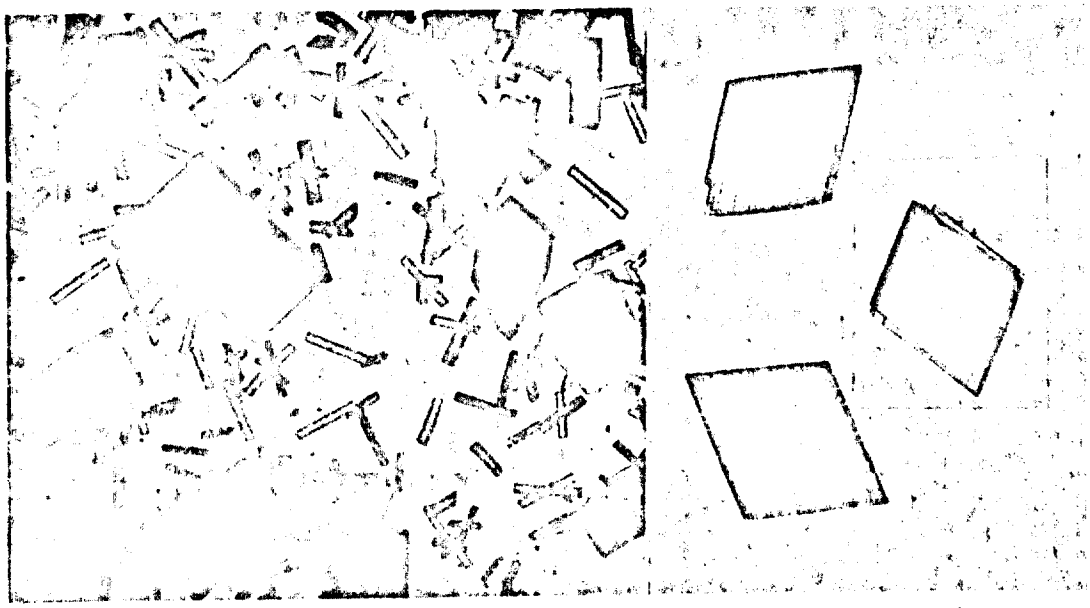

FIG. 2. Crystals of the BC and DBC coenzymes. *A* shows the BC coenzyme obtained by slow evaporation of an aqueous solution at 4°. The crystals were washed with acetone and ether and photographed in the dry state. Magnification 340 ×. *B* shows the DBC coenzyme crystallized from 87.5% acetone at 4°. The crystals were photographed in the mother liquor. Magnification 63 ×.

TABLE II

Isolation of benzimidazolylcobamide coenzyme from C. tetanomorphum

Starting material: 3.86 kg of cell paste.

Step	Volume of solution	Quantity of coenzyme*
	ml	*μmoles*
1. Ethanol extraction	1,200	68.5
2. Dowex 50-Na^+ treatment	1,800	72.6
3. Dowex 2-OH^- treatment	2,200	75.5
4. Phenol extraction	70	59.4
5. Peak from Dowex 50, pH 3 column	284	53.0
6. Phenol extraction	8	48.4
7. Crystallization		41

* Estimated by the spectrophotometric coenzyme assay by comparison with a purified sample of benzimidazolylcobamide coenzyme previously isolated. The apparent increase in the amount of coenzyme in Steps 2 and 3 is attributable to errors in the coenzyme assay caused by interfering substances in the cruder fractions.

washed with 1 ml of 90% acetone, 1 ml of 100% acetone, and finally with several milliliters of ethyl ether. The solid material was placed in a vacuum desiccator for a few minutes to remove ether. The resulting product, which contained 11.8% moisture removable by prolonged drying under reduced pressure at 4° over P_2O_5, weighed 68.7 mg and was estimated to contain 38.2 μmoles of coenzyme. An additional 3 to 4 μmoles of crystalline coenzyme were later recovered from the mother liquor of the first crop of crystals.

Table II gives data on the volume of solution and amounts of coenzyme at each step in the isolation.

DBC Coenzyme—This coenzyme has been isolated both from *C. tetanomorphum* grown in the presence of 5,6-dimethylbenzimidazole and from propionic acid bacteria (*P. shermanii* or *P. freudenreichii*) grown without addition of dimethylbenzimidazole to the medium. Since propionic acid bacteria are a richer source of DBC coenzyme than the clostridium, and the same methods are applicable to both organisms, only the coenzyme isolation from *P. shermanii* will be described.

Four kilograms of moist cells of *P. shermanii* were used. The first four steps in the isolation procedure were done as previously described. The aqueous solution obtained by phenol extraction and displacement of the active material back into water contained 440 μmoles of coenzyme in 269 ml. The amount of coenzyme was determined by activity assay on the assumption that the DBC coenzyme was the only coenzyme present.

The aqueous solution was diluted to 600 ml and was acidified to pH 3.0 with 1 N HCl at 0° with continuous stirring. Approximately 35 ml of acid were required. The solution was then diluted to 2 liters to reduce the salt concentration to about 0.01 M.

The diluted solution was passed into a 2 cm diameter × 80 cm high column of analytical grade Dowex 50W, pH 3, 2% cross linked, 200 to 400 mesh. The sample was washed into the column with a little water and then the column was eluted successively with 0.03 M sodium acetate, pH 5.5 (Fractions 80 to 295), and 0.05 M sodium acetate, pH 6.4 (Fractions 296 to 440). The "pass through" and eluate were collected in 10-minute fractions with a volume of approximately 25 ml. The course of the elution was followed by absorbancy measurements at 260 mμ. After the appearance of several minor absorbancy peaks, containing yellow, orange, or red components but no coenzyme activity, the red coenzyme was eluted by the pH 6.4 buffer in a prominent absorbancy peak between Fractions 336 and 380 (Fig. 3). At least two red compounds, probably hydroxocobalamin and factor B, remained on the column after elution of the coenzyme.

Determinations of visible and ultraviolet absorption spectra and coenzyme activities of peak fractions indicated the major component was DBC coenzyme, slightly contaminated in the end fractions by small amounts of other cobamide coenzymes. Fractions 341 to 370, containing 415 μmoles of coenzyme in 840 ml, were combined and extracted three times with, successively, 120, 50, and 40 ml of 92% aqueous phenol. After washing the combined phenol phase twice with 30 ml of water, the 158 ml of aqueous phenol containing the coenzyme were diluted with 3 volumes of ethyl ether and 1 volume of acetone and extracted twice with 20 ml and twice with 10 ml of water. The combined aqueous phase, containing the coenzyme, was washed twice with 20 ml of ether to remove phenol and was then aerated with N_2 to remove dissolved ether. The final solution was intensely red and contained 358 μmoles of coenzyme, estimated by its absorbancy at 522 mμ, in 69 ml.

The first crystals of DBC coenzyme were obtained by adding 2.1 ml of acetone to 0.3 ml of the above solution, scratching the walls of the tube, and leaving the solution at 3°. After 3 days small clusters of crystals were found on the walls of the tube. Crystallization was also induced in another sample by allowing an aqueous solution of the coenzyme to concentrate slowly under reduced pressure at 3° for several days.

After several trials with different methods of crystallization, the following procedure was found to be convenient and effective.

The aqueous solution of the coenzyme was concentrated, if necessary, under reduced pressure at 4° over sulfuric acid until it was 0.005 to 0.01 M. Any crystals formed by excessive concentration were dissolved in a minimum volume of water. To 20 ml of the concentrated aqueous coenzyme solution in a glass-stoppered flask were added 100 ml (5 volumes) of acetone. After standing at 4° for 2 to 3 hours the solution was centrifuged for 10 minutes at 10,000 × *g* in stainless steel tubes to remove

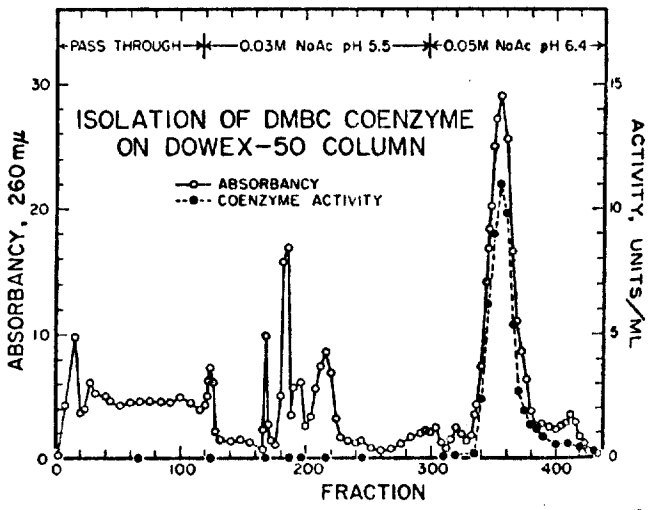

FIG. 3. Isolation of DMBC (DBC) coenzyme on a Dowex 50W column.

any amorphous precipitate that was present. The clear supernatant solution was seeded with a microscopic amount of crystalline DBC coenzyme and was allowed to crystallize over night at 4°. Dark red crystals formed on the walls and bottom of the container (Fig. 2*B*). Usually one-third to one-half of the coenzyme crystallized in this time. Another 40 ml (2 volumes) of acetone were added and the solution was left 4 or 5 days to crystallize. The course of crystallization was followed by measuring the absorbancy of the solution at 522 mμ. The absorbancy gradually fell to 0.25, corresponding to a concentration of 3×10^{-5} M. With an initial coenzyme concentration of 0.005 M, this corresponded to the crystallization of about 94% of the total cobamide. The supernatant solution was decanted and saved for recovery of a second crop of crystals. The crystals were washed twice with a few milliliters of cold 90% acetone, twice with 10% acetone, and twice with ethyl ether. During the acetone and ether wash most of the crystals separated from the glass and after removal of residual ether with a gentle stream of air, they were easily transferred to a weighing bottle. The coenzyme can be readily recrystallized by the same method.

Table III summarizes the data on the isolation of the DBC coenzyme.

With the use of the above crystallization method, 554 mg of crystalline DBC coenzyme were obtained in the first crop and an additional 24 mg were obtained in a second crop, once recrystallized. Since the crystals contained 8.2% of moisture (see below) and since the molecular weight of the dry coenzyme was estimated to be approximately 1660, the total yield of crystalline coenzyme corresponded to 320 μmoles, or 80 μmoles per kg of starting cell paste.

TABLE III

Isolation of 5,6-dimethylbenzimidazolylcobamide coenzyme from P. shermanii

Starting material: 4.0 kg of cell paste.

Step	Volume of solution	Quantity of coenzyme
	ml	*μmoles*
1. Ethanol extraction	3,000	not determined
2. Dowex 50-Na^+ treatment	3,450	not determined
3. Dowex 2-OH^- treatment	3,940	520
4. Phenol extraction	269	440
5. Peak from Dowex 50, pH 3 column	840	415
6. Phenol extraction	69	358
7. Crystallization		321

Properties of Coenzymes

Crystal Form and Color—The BC coenzyme crystallizes readily from water as small (<0.2 mm long) six-sided prisms with four rectangular and two diamond-shaped faces or as very small needles with blunt ends (Fig. 2*A*). The larger crystals are formed on the glass walls of the container near the surface of the liquid. Even with slow evaporation of an aqueous solution, much of the solid that forms is microcrystalline. Addition of 85% acetone to an aqueous solution of the coenzyme also gives well formed but very small crystals. No method of recrystallisation has been found that consistently gives the larger perfect crystals such as are readily obtained with the DBC coenzyme.

As previously mentioned, the crystals of the BC coenzyme are conspicuously pleochroic, appearing either yellow or red on the diamond faces depending on the angle of observation. The prism faces are always dark red. No detailed crystallographic study has been made of the BC coenzyme.

The DBC coenzyme crystallizes less readily from water but much more readily from acetone-water solutions than the BC coenzyme. Two distinct types of DBC coenzyme crystals have been observed to form in acetone-water. Most commonly the coenzyme crystallizes in the shape of flattened diamonds that occur singly or in small aggregates (Fig. 2*B*). On a few occasions, however, rosettes of very small, radiating needle-shaped crystals have been observed. These appear to be more soluble than the larger diamond-shaped crystals because on long standing in contact with the mother liquor, they gradually disappear and are replaced by the latter.

Crystals of the DBC coenzyme differ from those of the BC coenzyme by the absence of any readily observable pleochroism. They are uniformly red in color. Pleochroism in this compound is only apparent when the crystals are viewed by polarized light.

A crystallographic study of the DBC coenzyme is reported by Mattern (7).

Homogeneity of Crystalline Coenzymes—Crystallinity is not necessarily an indication of purity of a cobamide since some crystalline preparations of cobamide vitamins have been shown by ionophoresis to contain more than one component (8). Consequently, further evidence of the homogeneity of the coenzymes was sought by the use of paper ionophoresis, paper chromatography, and a solubility test.

Paper ionophoresis has been done with Whatman No. 1 paper with acid, neutral, and alkaline solvents. The acidic solvent, consisting of 0.5 M acetic acid, gives the greatest mobility, with both coenzymes. When 0.02 μmole of DBC coenzyme is used, the material moves as a single reddish-orange and ultraviolet-absorbing spot toward the cathode at a rate of approximately 12.8 cm per hour in a potential gradient of 40 volts per cm. Under the same conditions the BC coenzyme moves a little faster, 13.8 cm per hour. No evidence of a second component could be observed with either coenzyme under any of the conditions tested.

Paper chromatography of the BC and DBC coenzymes on Whatman No. 1 filter paper by the descending method, with a *sec*-butanol-glacial acetic acid-water (100:3:50 by volume) solvent for 20 hours at room temperature, showed the presence of only one visibly colored or ultraviolet-absorbing spot in each preparation. The R_F values were 0.16 and 0.22, respectively, for the BC and DBC coenzymes. By comparison on the same chromatogram, the R_F values of cyanocobalamin and the AC coenzyme were 0.34 and 0.09, respectively.

The solubility test for purity consisted of adding successive amounts of crystalline DBC coenzyme to water until the total quantity exceeded the solubility of the coenzyme by about 60%. After each addition, the solution was shaken until equilibrium was reached and the absorbancy of the solution at 260 mμ was determined. Fig. 4 shows that no significant increase in absorbancy occurred after the solution became saturated with coenzyme. This indicates that the crystalline DBC coenzyme contains less than 2% of ultraviolet light-absorbing impurities.

Stability—The dry crystalline coenzymes are moderately stable. No changes in spectrum or activity have been detected in samples stored several months at −10° or several days at room temperature. Solutions of the coenzymes appear to be most

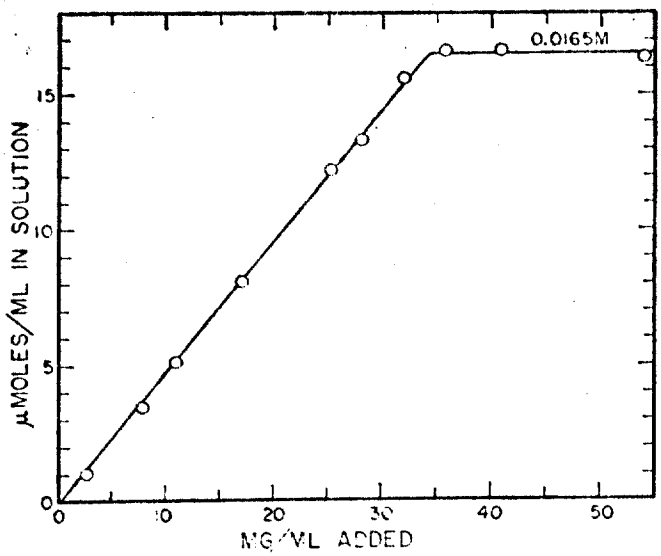

FIG. 4. Solubility test of homogeneity of the DBC coenzyme. Progressively larger amounts of crystalline DBC coenzyme were added to 1 ml of water at 24° ± 0.5°. After stirring the solution for 15 minutes and centrifuging down any remaining solid material, aliquots of 5 μl were diluted to 1.00 ml in 0.01 M KPO_4 buffer, pH 6.8, and the concentration of the original solution calculated from the absorbancy of the diluted sample at 260 mμ.

stable at pH 6 to 7. Neither coenzyme is appreciably inactivated by heating for 20 minutes at 100° in a 0.01 M sodium acetate buffer, pH 6.0, or by storage in dilute neutral solution for several months at −10°. As previously noted, the BC and DBC coenzymes are more stable under acid conditions than the AC coenzyme (2). Nevertheless, heating in 0.07 N HCl at 85° causes slow inactivation; in one experiment the BC coenzyme ost 8% of its activity during 5 minutes under these conditions.

TABLE IV

Solubility of benzimidazolylcobamide and 5,6-dimethylbenzimidazolylcobamide coenzymes

Finely powdered crystalline coenzyme was allowed to remain in contact with the indicated solvents, with occasional shaking, until the absorbancy of the supernatant solution became constant. This required 1 to 2 hours at room temperature and 1 to several days in the refrigerator. Coenzyme concentration was calculated from absorbancy readings at 519 mμ and 522 mμ for the BC and DBC coenzymes, respectively. The temperature indicated in brackets is that when the last two samples were taken for absorbancy readings. It is accurate to ±1°. The concentration of the acetone-water solutions is expressed in volumes % of acetone.

Coenzyme	Water	Acetone-water	
		83.3%	87.5%
	μmoles/ml	μmole/ml	μmole/ml
BC	1.23 (22°) 0.50 (1°)	0.077 (1°)	0.015 (1°)
DBC	16.4 (24°) 6.4 (1°)	0.052 (1°)	0.0085 (1°)

The great instability of solutions of the BC and DBC coenzymes under exposure to light or cyanide ion has already been reported (2). Either treatment causes a loss of activity and a dramatic change in the adsorption spectrum. The rates of decomposition increase with light intensity or cyanide ion concentration. These effects will be discussed more fully in a subsequent paper.

Solubility—The coenzymes show a solubility pattern similar to that of cyanocobalamin. They are rather soluble in water,

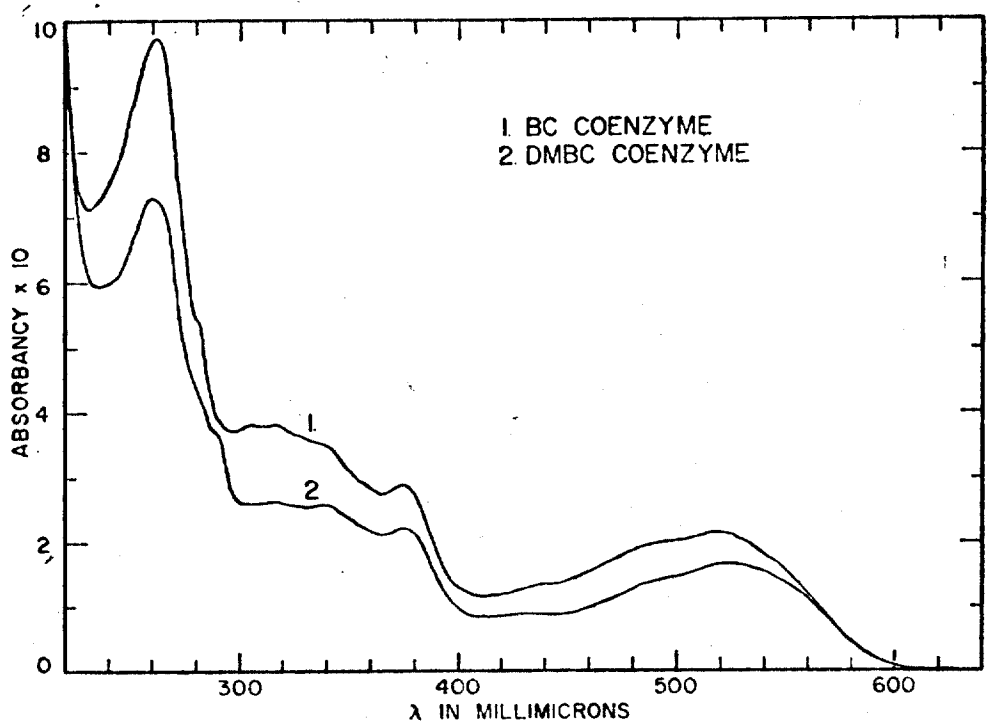

FIG. 5. Absorption spectra of 2.80×10^{-6} M BC coenzyme and 2.10×10^{-6} M DMBC (DBC) coenzyme in 0.01 M potassium phosphate buffer, pH 6.8.

ethanol, and phenol, but insoluble in acetone, ether, dichlorethylene, dioxane, and other relatively nonpolar solvents.

Data on the solubility of the coenzymes in water and acetone-water solutions are given in Table IV. Noteworthy is the rather striking difference between the solubility of the two coenzymes in water at room temperature. The relative ease with which the BC coenzyme can be crystallized from water is a reflection of its lower solubility in this solvent.

Absorption Spectra—Fig. 5 shows absorption spectra of solutions of crystalline BC and DBC coenzymes. The compounds have very similar spectra. The most distinctive difference between the two spectra is the position of the rather inconspicuous inflection on the side of the main absorbancy peak. With the BC coenzyme, this inflection comes at 280 mμ, whereas with the DBC coenzyme it is located at 288 mμ. There are also some other small differences between the two spectra. The BC coenzyme has maxima at 261 and 519 mμ whereas the corresponding maxima of the DBC coenzyme are at 260 and 522 mμ. The BC coenzyme also has an inconspicuous maximum at about 305 mμ which is lacking in the other compound. Both of these coenzymes differ from the AC coenzyme by their much redder color in neutral solution, by the position of the broad maximum in the visible region close to 520 mμ rather than at 458 mμ, and by other features.

Molar Extinction Coefficients—Treatment of any one of the cobamide coenzymes with 0.1 M KCN for 60 minutes at 25° causes the formation of a product with an absorption spectrum above 350 mμ characteristic of the dicyanocobamides (1, 2). By making the assumption (see below) that the molar extinction coefficient of the 367 mμ peak of the product formed in this way from a cobamide coenzyme is the same as that of cyanocobalamin in the presence of 0.1 M KCN, namely 30.4×10^{-6} cm² per mole, the molar concentration of a coenzyme solution can be calculated. Knowing the concentration, the molar extinction coefficients of the coenzyme at various wave lengths can be estimated from its absorption spectrum in the absence of cyanide.

Table V gives the millimolar extinction coefficients of the BC, DBC, and AC coenzymes in neutral solution and in 0.1 M KCN, estimated by the above method. A comparison of the extinction coefficients of the three coenzymes in the presence of cyanide at 350, 367, 540, and 579 mμ, demonstrates that their spectra are very similar if not identical in this region. Cyanocobalamin in the presence of 0.1 M KCN also has the same spectrum above 350 mμ. The virtual identity of the spectra of all these compounds appears to be a consequence of the fact that excess cyanide ion displaces the purine or benzimidazole moieties from the cobalto-corrin to form its dicyano derivative. This structure is evidently entirely responsible for the spectrum above 350 mμ. The nucleotide bases absorb light of shorter wave lengths and only influence the spectrum above 350 mμ when they are able to form a coordinate linkage with the cobalt. Under these circumstances, the assumption that the molar extinction coefficients of the cobamide coenzymes are the same as that of cyanocobalamin at 367 mμ in 0.1 M KCN appears to be justified. Further evidence for the approximate correctness of this assumption is provided by analytical data on the phosphate and cobalt contents of the coenzymes (see below).

Assuming the molar extinction coefficients given in Table V to be correct, the molecular weight of a coenzyme can be determined by measuring the absorbancy of a solution containing a known weight of the dry crystalline compound. Samples of the DBC coenzyme were dried for 2 hours in a vacuum over silica gel at 56.5, 78.5, and 100°. The percentage weight loss was the same within experimental error at all three temperatures, the average being 8.2%. Solutions of the dried samples showed the same spectrum and the same specific activity in the coenzyme assay as the undried coenzyme. The molecular weight calculated from absorbancy measurements at six wave lengths on the three coenzyme samples dried at different temperatures was found to be 1660 ± 20.

The molecular weight of the BC coenzyme was estimated by the same method, with a sample of crystalline coenzyme dried to constant weight during 6 weeks in a vacuum over P_2O_5 at 4°, to be 1,610 ± 20.

Elementary Analysis—Cobalt was identified in both the BC and DBC coenzymes by means of an x-ray fluorescence spectrometer (3). Quantitative cobalt estimations by the nitrosocresol method (9), after wet digestion with a mixture of nitric, perchloric, and sulfuric acids (10), gave values of 0.93 and 0.95 atom of cobalt per mole of BC and DBC coenzyme, respectively, estimated spectrophotometrically.

Total phosphate determinations by the method of Fiske and SubbaRow (11) after digestion with nitric and sulfuric acids and

TABLE V

Millimolar extinction coefficients of cobamide coenzymes in presence and absence of cyanide

The BC and DBC coenzymes used for these determinations were the best crystalline preparations; the AC coenzyme was a highly purified noncrystalline preparation. All values are based on $E_{1\text{ cm}}^{0.001\text{ M}}$ at 367 mμ of 30.4×10^6 cm² per mole for the dicyanide derivative. The spectra of the coenzymes in 0.1 M KCN were taken after the reaction with cyanide was complete, as indicated by maximal formation of the peaks at 367, 540, and 579 mμ.

λ	$E_{1\text{ cm}}^{0.001\text{ M}} \times 10^6$ cm² per mole					
	BC coenzyme		DBC coenzyme		AC coenzyme	
	0.01 M KPO_4 pH 6.7	0.1 M KCN pH ~10	0.01 M KPO_4 pH 6.7	0.1 M KCN pH ~10	0.01 M KPO_4 pH 6.7	0.1 M KCN pH ~10
mμ						
230	25.2					
233			26.2			
239					26.7	
260			34.7			
261	34.7					
263					45.3	
264		28.4				
268				22.7		29.0
280	19.1					
288			18.1			
303		12.8			21.6	
306	13.3			9.65		10.6
315	13.4		13.2			
327		6.9		4.3		
340	12.3		12.3			
350		14.9		14.6		14.7
367		30.4		30.4		30.4
375	10.2		10.9		9.0	
458					8.75	
519	7.65					
522			8.0			
540		8.5		8.6		8.5
579		10.0		10.1		10.0

hydrogen peroxide showed the presence of 0.97 and 0.98 atom of phosphorus per mole of BC and DBC coenzyme, respectively, estimated spectrophotometrically.

No sulfur could be detected in 1.1 μmoles of DBC coenzyme by the nitroprusside test (6) after sodium fusion. Under identical conditions 0.3 μmole of thiamine gave a strong positive test for sulfur.

Elementary analysis by a commercial laboratory of a sample of crystalline DBC coenzyme dried in a vacuum for 2 hours at 56.5° gave the following results: C 53.5%, H 6.9%, N 14.9%. On the basis of the above analytical data and the assumption (see below) that the DBC coenzyme contains 18 atoms of nitrogen, its empirical formula can be calculated to be approximately $C_{74}H_{114}O_{21}N_{18}PCo$. This formula gives a molecular weight of 1701 which is 2.6% higher than the molecular weight of 1,660 calculated from dry weight determinations and the molar extinction coefficients given in Table V. If we assume the lower value is more nearly correct, we must reduce the number of oxygen atoms in the empirical formula by one or two and possibly also reduce the number of C and H atoms. On this basis the formula $C_{72-74}H_{112-114}O_{19-20}N_{18}PCo$ appears to cover the range of possibilities. The corresponding minimal and maximal molecular weights are 1636 and 1685, respectively.

No C, H, and N analyses have been done on the BC coenzyme.

Components—No cyanide could be detected in either coenzyme by the method of Boxer and Rickards (12).

Adenine was identified and estimated semiquantitatively after acid hydrolysis of the BC coenzyme in the following manner.

A sample containing 0.11 μmole of the coenzyme was hydrolyzed for 60 minutes in 1 N HCl at 100°. After cooling, the solution was concentrated to dryness and the residue was dissolved in a little 60% (volume per volume) ethanol. The ethanol extract was divided into two portions to one of which was added some adenine as an internal control. Both solutions and separate adenine, adenosine, and adenylic acid standards were chromatographed on Whatman No. 1 paper by the ascending method with a solvent containing 50% (volume per volume) ethanol and 50% aqueous 0.5 M sodium acetate, pH 7.2. When the paper was examined by ultraviolet light, a quenching spot appeared in the hydrolyzed sample at R_F 0.63 that did not separate from the added adenine, but readily separated from adenylic acid (R_F 0.56) and adenosine (R_F 0.70). The quenching spots at R_F 0.63 were eluted and their absorption spectra determined. The unknown showed a typical adenine spectrum in neutral solution with an absorbancy maximum at 260 mμ which shifted to 267 mμ on addition of alkali. From the absorbancy at 260 mμ in neutral solution, corrected for a suitable paper blank and for a 60% recovery of an adenine standard, the yield of adenine was estimated to be 1.0 ± 0.2 μmole per μmole of BC coenzyme hydrolyzed.

The presence of adenine in an acid hydrolysate of the DBC coenzyme was established by similar methods. In this case, a colorless compound with an absorbancy maximum at 260 mμ was separated from the other products of acid hydrolysis by means of a Dowex 50W, pH 3 column. The compound eluted with 0.03 M sodium acetate, pH 6, in a well defined peak which was only slightly contaminated by a faster moving red compound. The elution rate of the colorless compound was like that of adenine. The hydrolysis product was further identified as adenine by its absorption spectrum in neutral and alkaline solution and by its R_F in paper chromatography. The yield of adenine, calculated from the absorbancy at 260 mμ of the elution peak fractions, was 0.92 mole per mole of DBC coenzyme.

Benzimidazole was shown to be a component of the BC coenzyme and 5,6-dimethylbenzimidazole of the DBC coenzyme. These compounds were separated from the corresponding coenzymes by acid hydrolysis, alkalinization of the hydrolysate, and extraction of the heterocyclic base with chloroform. After removal of the organic solvent, the base was identified by its absorption spectrum, its fluorescence spectrum, and by paper chromatography.

The experimental details of the identification of benzimidazole in the BC coenzyme are as follows:

A sample containing 0.17 μmole of BC coenzyme was dissolved in 0.6 ml of 6 N HCl and heated in an evacuated and sealed tube for 18 hours at 150°. The solution was transferred to a 10-ml beaker and dried under a stream of warm air. A little water was added and the solution was again evaporated to dryness to remove excess HCl. The residue was dissolved in 1 ml of water, alkalinized with 0.01 ml of 10 N KOH, and shaken with 12 ml of chloroform. The organic phase was transferred to a small beaker and the chloroform was removed by a stream of warm air. The residue was dissolved in a little ethanol, and an aliquot was used for ascending paper chromatography. A blue fluorescent spot corresponding to benzimidazole was observed at R_F 0.78, with *sec*-butanol-acetic acid-water, 100:3:50 by volume, as the developing solvent, and at R_F 0.72 with 0.1 N acetic acid as a solvent. Another aliquot was evaporated to dryness, dissolved in 0.1 N acetic acid, and its ultraviolet absorption spectrum determined. The spectrum was identical with that of benzimidazole with characteristic absorption maxima at 239, 260, 266, and 273 mμ, and a sharp minimum at 270 mμ. The fluorescence of the hydrolysis product was compared with that of benzimidazole with an Aminco spectrophotofluorometer. Both samples showed a strong fluorescence in 0.1 N acetic acid that was maximally activated by light at 272 mμ and was most intense at 365 mμ. The fluorescence of both samples was quenched by addition of 0.1 N HCl.

The yield of benzimidazole, determined from the absorbancy at 273 mμ and the corresponding molar extinction coefficient of 8100 cm² per mole, was 0.80 mole per mole of coenzyme.

In the quantitative determination of benzimidazole or dimethylbenzimidazole, a standard of the appropriate benzimidazole and a reagent blank were carried through the entire extraction and estimation procedure and used to determine appropriate corrections for the unknown sample. It was also determined that benzimidazoles are not appreciably decomposed under the conditions of acid hydrolysis employed.

5,6-Dimethylbenzimidazole was identified as a product of acid hydrolysis of the DBC coenzyme by the same methods. The R_F of 5,6-dimethylbenzimidazole and the unknown was found to be 0.82 in *sec*-butanol-acetic acid-water and 0.60 in 0.1 N acetic acid. The spectra of both samples had absorption maxima at 245, 273, and 283 and a sharp minimum at 280 mμ. The fluorescence of both samples was maximally activated by light at 285 mμ and was most intense at 380 mμ.

The yield of 5,6-dimethylbenzimidazole formed by acid hydrolysis of the DBC coenzyme, calculated from its absorbancy in 0.01 N HCl at 283 mμ and the corresponding molar extinction coefficient of 8,100 cm² per mole, was 0.73 mole per mole of coenzyme.

Relative Coenzyme Activities—Activities of the cobamide co-

nzymes in catalyzing the conversion of glutamate to β-methylaspartate can be compared either at low or at high coenzyme concentrations. A comparison at low concentrations, where the reaction rate is proportional to concentration, shows that the activity per mole is highest for the BC coenzyme, intermediate for the AC coenzyme, and lowest for the DBC coenzyme (Table VI). This difference in molar activities could be the result of differences either in the affinities (K_m values) of the coenzymes for the enzyme system or in their potential maximum activities (V_{max} values). Determination of the apparent K_m values in this multienzyme system shows that the relative molar activities are largely a reflection of differences in the K_m values (Table VI). The product $K_m \times$ molar activity (*Column 4*) is almost a constant for the three coenzymes, indicating that the molar activities are inversely proportional to the affinities. The maximal activities of the three coenzymes are very similar (*Column 5*).

Growth Factor Activity—Activity was measured in the *E. coli* B_{12}-requiring mutant and *Ochromonas malhamensis* tests as previously described (3). In the *E. coli* test, both the DBC and BC coenzymes showed 100 ± 20% of the activity of cyanocobalamin, on a molar basis. In the Ochromonas test, the DBC coenzyme showed 90 ± 20% and the BC coenzyme showed 45 ± 10% of the activity of cyanocobalamin on a molar basis. The value obtained with the BC coenzyme is similar to that reported for the benzimidazole analogue of cyanocobalamin (13).

DISCUSSION

The methods described for the isolation of the coenzymes are ather effective since they result in an over-all purification from ,000-fold with the DBC coenzyme to 8,000-fold with the BC coenzyme, and an over-all yield of crystalline products ranging from 54 to 62% of the extracted coenzymes. It is worth noting that the selected coenzyme peak fractions from the Dowex 50W, pH 3 column contain essentially pure coenzyme, since the specific activity of the coenzyme in these fractions is virtually the same as that of the final crystalline product. Consequently for some purposes the second phenol extraction and crystallization steps are unnecessary.

The DBC coenzyme content of *P. shermanii* (130 μmoles per kg of wet cells) is approximately 6.5 times greater than the DBC or BC coenzyme content of *C. tetanomorphum* (20 μmoles per kg). However, since the molar activity of the BC coenzyme in the *C. tetanomorphum* assay is about 73 times that of the DBC coenzyme, the total cobamide coenzyme activity per kg is about 11 times greater in *C. tetanomorphum* cells grown in the presence of benzimidazole than in *P. shermanii* cells. The latter are grown without addition of 5,6-dimethylbenzimidazole to the medium since the organism synthesizes this compound in adequate amounts.

The once recrystallized DBC coenzyme, carefully protected from light, appears to be at least 98% pure. This estimate is based upon the results of the solubility test for homogeneity and on the failure to detect colored impurities separable by paper ionophoresis or chromatography. The purity of the crystalline BC coenzyme has not been determined as carefully, but the failure to detect color impurities by ionophoresis or chromatography indicates that the coenzyme is at least 95% pure.

The analytical data demonstrate the presence of approximately one mole each of cobalt, phosphorus, and either benzimidazole or 5,6-dimethylbenzimidazole per mole of coenzyme. These results clearly establish a chemical relation of these compounds

TABLE VI

Affinities and relative activities of cobamide coenzymes

Apparent K_m values were estimated from a plot of S/V against S where V is the rate of absorbancy change and S is the coenzyme concentration. The experimental conditions were those used in the spectrophotometric coenzyme activity assay (3). The micromolar activity was obtained by dividing the observed activities at low ($\leq 0.4\ K_m$) coenzyme concentration by the number of micromoles of coenzyme per ml, determined from the absorbancy (A) at a suitable wave length. Typical and directly comparable absolute values are given in *Column 3*. The V_{max} values were calculated from the rates of absorbancy change under the conditions of the coenzyme activity assay, except that each coenzyme was used at a concentration 3.1 times its K_m value, and from the relation $V_{max} = V\left(\frac{[CoE]/K_m + 1}{[CoE]/K_m}\right)$. Crystalline preparations of BC and DBC coenzymes were used; the AC coenzyme was a highly purified noncrystalline preparation.

Coenzyme	(a) K_m	(b) Micromolar activity	(a) × (b)	V_{max}
	M	ΔA/*min./μmole*	× 10^{-4}	ΔA/*min.*
BC	2.4×10^{-7}	800	1.9	0.24
AC	1.4×10^{-6}	160	2.2	0.21
DBC	1.8×10^{-6}	11	2.0	0.17

with cyanocobalamin and its benzimidazole analogue, a relation previously indicated by a study of the absorption spectra of products formed by the action of light or cyanide ion on the coenzymes (2). The main differences in composition so far observed between the BC and DBC coenzymes and the corresponding vitamins are the absence of cyanide and the presence of an adenine moiety in the coenzymes. The AC coenzyme and pseudovitamin B_{12} differ in the same ways (3). The extra adenine moiety appears to be an essential structural feature of the cobamide coenzymes.

Various types of evidence that will be presented in a later paper show that hydroxocobalamin can be formed from the DBC coenzyme. If we tentatively accept this as a fact, then the coenzyme must contain the hydroxocobalamin structure with a molecular weight of about 1355, and an adenine moiety with a molecular weight of about 135. If these were the only components, the molecular weight of the coenzyme would be close to 1490. The experimentally determined molecular weight, calculated from the dry weight of the crystalline DBC coenzyme and either its phosphorus content or its estimated molar extinction coefficient, is approximately 1660. This leaves roughly 170 g not accounted for by the known components and indicates that the coenzyme contains an additional compound. This compound cannot contain cobalt or phosphorus since these are already accounted for by the hydroxocobalamin structure. The unknown compound also probably does not contain nitrogen, since the 18 nitrogen atoms present in hydroxocobalamin and adenine satisfactorily account for the elementary analysis and a molecular weight between 1640 and 1700. Consequently it probably contains only carbon, hydrogen, and oxygen. The determined empirical formula of the DBC coenzyme, $C_{72\text{-}73}H_{112\text{-}113}O_{19\text{-}20}N_{18}PCo$, in fact has 5 to 8 carbon atoms, 19 to 22 hydrogen atoms, and 5 to 6 oxygen atoms in excess of those contributed by the known components. Some of the hydrogen and oxygen may be present as water of hydration not removed by drying at 100°, but all of

the carbon and much of the hydrogen and oxygen must be present in the unidentified moiety.

All three of the cobamide coenzymes so far described are equally active in catalyzing the conversion of glutamate to β-methylaspartate, when tested at saturating coenzyme concentrations. However, the coenzymes differ greatly in their affinities for the enzyme system; the BC coenzyme has by far the highest and the DBC coenzyme the lowest affinity. These differences determine the minimum concentration at which the coenzymes can be detected by the enzymatic assay. For example, the BC coenzyme can be estimated at a level of 10^{-5} μmole per ml of the assay mixture, whereas approximately 10^{-3} μmole of DBC coenzyme is required. The AC coenzyme and several other homologous coenzymes that have been recently isolated are intermediate between these two coenzymes in affinity for the enzyme system.

The identity of the V_{max} values of the AC, BC, and DBC coenzymes clearly indicates that the 6-membered rings of the nucleotide adenine and benzimidazoles and their substituent groups are not directly involved in the catalytic activity of the coenzymes.

The activities of the DBC and BC coenzymes as growth factors for the *E. coli* mutant and for *Ochromonas malhamensis* are approximately the same as those of the corresponding vitamins. This could mean either that the vitamins are converted quantitatively to the coenzymes by these organisms or vice versa. Further experiments are needed to elucidate this point.

SUMMARY

Methods for isolation of benzimidazolylcobamide and 5,6-dimethylbenzimidazolylcobamide coenzymes, active in catalyzing the interconversion of glutamate and β-methylaspartate, have been described. The crystalline benzimidazolylcobamide coenzyme was obtained from *Clostridium tetanomorphum* in a yield of 10.6 μmoles per kg of moist cells, the crystalline 5,6-dimethylbenzimidazolylcobamide coenzyme from *Propionibacterium shermanii* in a yield of 80 μmoles per kg. The coenzymes contain approximately one mole each of cobalt, phosphorus, and heterocyclic base, either benzimidazole in the benzimidazolylcobamide coenzyme or 5,6-dimethylbenzimidazole in the 5,6-dimethylbenzimidazolylcobamide coenzyme. In addition, both coenzymes contain one mole of adenine which is not present in the corresponding cobamide vitamins. The coenzymes differ markedly in their affinities for the enzyme system, but have the same maximal activities. Several physical, chemical, and biological properties of the coenzymes are described.

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Cyanocobalamin (Vitamin B_{12})*

A Study of the Stability of Cyanocobalamin and Ascorbic Acid in Liquid Formulations

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The effect of *p*H on the stability of solutions of cyanocobalamin and ascorbic acid, alone, and in combinations, is reported. The effect of a contaminant complexing agent at one concentration on aqueous solutions of these vitamins is also reported. The stability of these vitamins, alone, and in combination, in various mixtures of propylene glycol, Sorbo, syrup, glycerin, glucose, and distilled water has been determined. The analytical methods employed for following the stability of cyanocobalamin include the U. S. P. method and the chemical method.

HERE IS A DEARTH of literature concerning the stability of vitamin B_{12} (1–5). The interest in the combination of this vitamin with ascorbic acid (6–8) justified a basic study of the incompatibility of cyanocobalamin with ascorbic acid (1). Preliminary investigations are reported evaluating the effect of *p*H, the effect of a trace-contaminant complexing agent, and the effect of various vehicles on the stability of cyanocobalamin and ascorbic acid, separately and in combination.

EXPERIMENTAL

The analytical procedures used for the determination of cyanocobalamin included both the chemical method (9–11) and the U. S. P. method (12). Ascorbic acid was determined by titration in a 1% oxalic acid solution with 0.1 *N* iodine using starch solution as the indicator. Generally, determinations were discontinued after about 50% decomposition had taken place.

The cyanocobalamin used in this laboratory was in the form of a 0.1% trituration with mannitol as the diluent. The ascorbic acid used in the study was of U. S. P. quality in a fine crystalline form. The water used as a solvent was distilled water redistilled from an all-glass still, unless otherwise specified. The other solvents, reagents, and salts were of official quality, unless otherwise identified. Containers, pipets, and all glassware used were thoroughly washed, rinsed several times with glass-distilled water, and air-dried before use.

The samples contained 10 ± 2 μg./cc. of cyanocobalamin and/or 50 ± 5 mg./cc. of ascorbic acid. Two-hundredths per cent of propylparaben was employed in all formulations as the preservative. After filtered (glass wool) solutions were subdivided into one-ounce flint glass, screw-capped bottles and stored in cardboard boxes at refrigerated (5°), elevated (40°), and room (25–30°) temperatures.

The Effect of pH. A series of solutions was prepared using 1 *M* sodium phosphate and 0.5 *M* citric acid buffer stock solutions (13). Solutions of ascorbic acid and cyanocobalamin were prepared at several *p*H levels between 2.2 and 7.0. Maximum stability for ascorbic acid was achieved at *p*H 6.3, which is in agreement with previous reports (14–16). Under the test conditions employed, which included protection from strong light, only the cyanocobalamin sample at *p*H 2.2 showed a loss (5%) after eight months at 40°. Maximum stability of a sample containing both ascorbic acid and cyanocobalamin was exhibited at *p*H 6.3. This finding is contrary to those reported for vitamin B_{12a} and ascorbic acid (2).

The Effect of a Complexing Agent.—Tetrasodium ethylenediaminetetraacetate[1] (NaEDTA) was used

TABLE I.—COMPOSITION OF VEHICLES USED AS SOLVENTS FOR CYANOCOBALAMIN AND ASCORBIC ACID

	Percentage by Volume					
Vehicle	Propylene Glycol	Glycerin	Glass Distilled Water	Syrup U. S. P.[a]	Sorbo[b]	Glucose
A	100	...	...	...	...	...
B	75	25	...	...	...	...
C	50	50	...	...	...	...
D	75	...	25	...	...	...
E	50	...	50	...	...	...
F	25	...	...	...	75	...
G	50	...	...	...	50	...
H	75	...	...	...	25	...
I	...	...	...	...	100	...
J	...	...	...	...	75	25
K	...	25	...	...	75	...
L	...	...	25	...	75	...
M	...	...	...	25	75	...
N	...	...	...	...	50	50
O	...	50	...	...	50	...
P	...	...	50	...	50	...
Q	...	...	...	50	50	...
R	...	75	...	...	25	...
S	...	...	75	...	25	...
T	...	...	...	75	25	...

[a] Syrup U. S. P. was prepared by the cold method.
[b] Sorbo is a 70% aqueous solution of *d*-sorbitol commercially available from the Atlas Powder Company.

[1] Tetrasodium ethylenediaminetetraacetate was graciously supplied by the Bersworth Chemical Co. under the trade name, Versene.

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TABLE II.—EFFECT OF TEMPERATURE ON STABILITY OF SOLUTIONS CONTAINING 50 ± 5 MILLIGRAMS ASCORBIC ACID PER CUBIC CENTIMETER

Vehicle	°C.[a]	Percentage of Ascorbic Acid Retained, Days: 10	30	60	90	120	150	180	210
A	5	...	100	100	100	...	100	100	...
	RT	100	100	100	89.0	...	72.0	59.0	...
	40	100	97.7	93.9	90.6	61.8	36.2	...	...
B	5	...	100	100	100	...	100	100	...
	RT	...	100	100	100	...	99.2	91.3	88.3
	40	100	96.5	92.5	92.0	90.6	...	...	...
C	5	...	100	100	100	...	100	...	100
	RT	...	98.0	...	98.0	96.0	92.0	...	93.2
	40	100	99.8	100	88.8	86.7	...	...	...
D	5	...	100	100	100	100	...	...	100
	RT	...	95.8	97.9	98.0	...	91.3	90.8	89.0
	40	98.0	94.0	91.3	91.3	90.8	88.8	84.3	84.0
E	5	...	100	100	100	100	...	...	100
	RT	...	100	95.3	...	97.3	94.5	93.1	90.0
	40	100	93.8	91.8	86.3	72.5	59.2	...	...
F	5	...	...	Gelled	...	...	...	...	...
	RT	...	92.3	92.3	90.9	91.8	...	89.1	...
	40	100	100	91.5	90.3	86.3	...	...	...
G	5	Gelled	...	...	...	...	...	...	...
	RT	...	92.7	91.8	90.9	...	82.1	79.8	...
	40	97.3	92.5	90.5	88.0	...	76.8	72.5	71.0
H	5	...	...	100	100	...	100	Ppt.[b]	...
	RT	...	100	99.2	...	95.7	96.0	94.0	90.2
	40	100	90.8	...	88.8	86.5	83.0	83.8	79.5
I	5	...	...	...	...	...	100	...	100
	RT	...	100	100	100	100	92.7	91.8	91.5
	40	100	87.0	...	76.8	71.8	64.8	55.8	52.3
J	5	...	98.0	98.0	98.0	98.0	98.0	98.0	98.0
	RT	...	...	...	...	95.5	92.5	85.5	85.5
	40	97.5	87.3	81.3	78.8	76.0	68.0	64.0	61.0
K	5	...	100	100	...	...	100	...	...
	RT	...	90.5	87.5	90.3	88.0	87.5	86.3	85.7
	40	97.5	94.3	92.3	89.3	86.5	80.8	78.8	74.3
L	5	...	100	100	100	...	100	...	...
	RT	...	95.5	95.4	...	94.2	...	93.0	91.0
	40	100	93.5	...	82.9	76.6	67.0	62.1	54.4
M	5	...	100	100	...	...	100	...	...
	RT	...	100	100	...	89.2	89.2	...	76.9
	40	97.3	92.0	85.0	...	81.2	75.3	...	59.2
N.	5	...	100	...	...	...	...	...	...
	RT	...	100	100	94.5	90.2	85.9	80.2	73.9
	40	100	93.0	86.7	79.0	74.0	73.0	59.5	...
O	5	...	100	100	...	...	100	100	...
	RT	...	98.2	98.4	97.8	...	95.3	...	92.1
	40	...	97.8	97.8	92.7	91.1	...	...	...
P	5	...	100	100	...	...	...	...	100
	RT	...	94.3	94.3	83.0	82.0	80.0	73.2	71.1
	40	100	87.0	73.8	61.3	49.0	37.8	...	...
Q	5	...	100	100	...	100	100	98.0	98.0
	RT	...	94.6	...	89.1	...	72.3	...	64.3
	40	89.0	87.5	81.5	78.2	73.3	68.7	58.2	52.5
R	5	100	...	100	...	...	...	...	...
	RT	100	...	98.9	98.0	97.4	97.0	...	95.1
	40	90.3	88.5	89.3	87.5	85.7	86.7	83.6	84.5
S	5	97.8	...	98.5	97.1	95.4	...	...	...
	RT	...	96.5	92.0	82.1	75.8	60.3	54.1	...
	40	96.3	82.8	67.0	52.6	32.5	...	...	...
T	5	100	100	100	100	100	...	...	...
	RT	...	95.1	...	90.1	84.0	80.3	70.0	51.2
	40	96.0	91.0	88.5	74.0	68.3	47.5	...	...

[a] RT = Room temperature (25–30°). [b] Ppt.—Precipitation occurred.

at a concentration of 0.05% (17). The complexing agent was effective in stabilizing a solution of ascorbic acid prepared with water distilled from a metal still (18).

Two aqueous solutions of cyanocobalamin were prepared containing NaEDTA. One solution was made acid (*p*H *ca.* 5) with 1 *N* sulfuric acid. The unneutralized solution had a *p*H of 10.3. There was about 50% decomposition of the cyanocobalamin in the unneutralized solution after one week at 40° and one month at room temperature. The *p*H of the decomposed solution remained at 10.3. The acid solution made acid exhibited no change in the cyanocobalamin content after storage for two months. The vitamin content was determined spectrophotometrically using a suitable blank.

o aqueous solutions of a mixture of cyano-
lamin and ascorbic acid were prepared. To
olution was added NaEDTA. Both solutions
a pH of about 2.5. After sixty days at 40°,
sample containing NaEDTA had retained
% of vitamin B_{12} and 72.3% of ascorbic acid
ared to 35.9% and 66.1%, respectively, in
other sample. The period of study was too
to make possible conclusions regarding the
stabilization noted. Investigations are being
inued. The cyanocobalamin content was de-
ined by the chemical method.

Effect of Various Vehicles.— Solutions of
ocobalamin and ascorbic acid were prepared
lying solvents most commonly used in vitamin
arations (Table I). Several of these vehicles
used to prepare solutions containing both
obalamin and ascorbic acid. The cyano-
lamin determinations were made by the chemi-
method. A high concentration of propylene
l with water, glycerin, or Sorbo, and a mixture
cerin and Sorbo were found to afford maximum
ility conditions for ascorbic acid (Table II).
ocobalamin was found to be stable in a wide
ty of solvents. It has questionable stability
gh concentrations of propylene glycol and in
nts containing glucose (Table III). A study
ble IV indicated that vehicle *C*, composed of
parts, by volume, of propylene glycol and
in, afforded maximum stability conditions for
ture of cyanocobalamin and ascorbic acid.

DISCUSSION

k 0.1% trituration in mannitol provided crystal-
yanocobalamin in a form which was con-
nt to weigh and handle. A stabilizing effect
solution of ascorbic acid by mannitol was
. The unsatisfactory use of liquefied phenol
P. as a preservative for cyanocobalamin has
confirmed (4).

he percentage of propylparaben in the finished
ons was not known, the excess having been
l out, but the amount present was apparently
e in inhibiting mold growth in all samples
study.

E III.—EFFECT OF TEMPERATURE ON STA-
OF SOLUTIONS CONTAINING 10 ± 2 MICRO-
OF CYANOCOBALAMIN PER CUBIC CENTIMETER

Vehicle	°C.[a]	Percentage of Cyanocobalamin Retained, Days 30	60
A	5	100	100
	RT	96.8	90.4
	40	88.6	65.4
D	5	100	100
	RT	100	88.2
	40	100	85.2
H	5	...	ppt.[b]
	RT	...	87.1
	40	...	82.0
J	5	83.6	...
	RT	77.2	...
	40	76.2	...
N	5	...	96.4
	RT	...	90.3
	40	...	88.9

[a] RT = Room temperature (25–30°).
[b] ppt.—Precipitation occurred.

Although the chemical method of analysis of cyanocobalamin may have indicated no decomposition, all vehicles containing mixtures of cyanocobalamin and ascorbic acid exhibited various color changes on storage. It is believed that the color changes were a result of ascorbic acid decomposition. The observation leading to this belief was based on the ability to reproduce the color changes exhibited by solutions of cyanocobalamin and ascorbic acid, undergoing decomposition on storage, by adding ascorbic acid solutions in various stages of decomposition to an undecomposed solution of cyanocobalamin. It is known that ascorbic acid solutions, initially colorless, decompose with the production of yellow to brown solutions, depending on the extent of decomposition.

Freshly prepared solutions of cyanocobalamin and ascorbic acid are pink due to the cyanocobalamin content; the color gradually changes, however, to orange and finally to brown. The rapidity of the color change is directly related to the rate of color development in a solution of ascorbic acid with the same vehicle. A color wheel shows that the colors red (or pink from the cyanocobalamin) and yellow (decomposed ascorbic acid solution) combine to form an orange color (color of solution of cyanocobalamin and ascorbic acid after storage). Since only after the color starts turning a deeper orange toward orange-brown is there any indication of a cyanocobalamin loss by the chemical method, it is suspected that the decomposition products of ascorbic acid play an important part in the decomposition of cyanocobalamin. Table V summarizes the color changes that occur in solutions of ascorbic acid and in solutions of mixtures of cyanocobalamin and ascorbic acid on storage. The colors appearing in the bottom row of the table may be produced by combining solutions with the colors indicated in the first two rows.

SUMMARY

The optimum *p*H for stability of ascorbic acid in solution was found to be above 6.0 but below 7.0. The optimum *p*H for cyanocobalamin is between 4.5 and 5.0. The most favorable *p*H for a mixture of cyanocobalamin and ascorbic acid appears to be between 6.0 and 7.0.

Tetrasodium ethylenediaminetetraacetate has a stabilizing effect on ascorbic acid in distilled water. Unless acidified, it has a deleterious effect on cyanocobalamin. A slight stabilization was noted at elevated temperature on the active ingredients when the complexing agent was included in a mixture of cyanocobalamin and ascorbic acid.

Ascorbic acid was found to be most stable in high concentrations of propylene glycol with distilled water, glycerin or Sorbo, and a mixture of glycerin and Sorbo. Cyanocobalamin was found to be stable in a wide variety of vehicles. It has questionable stability in high concentrations of propylene glycol and in vehicles contain-

TABLE IV. EFFECT OF TEMPERATURE ON STABILITY OF SOLUTIONS CONTAINING 10 ± 2 MICROGRAMS CYANOCOBALAMIN AND 50 ± 5 MILLIGRAMS OF ASCORBIC ACID PER CUBIC CENTIMETER

Vehicle	°C.[a]	Percentage Retained, Days: 30		60		90		120		150		180
		B_{12}[b]	AA[c]	B_{12}	AA	B_{12}	AA	B_{12}	AA	B_{12}	AA	B_{12}
A	5	100	100	100	100	100	100	100	99.3	100	96.2	100
	RT	...	96.5	99.6	93.8	90.3	87.5	...	83.5	77.3	81.0	...
	40	100	95.8	90.3	95.9	85.0	89.8	76.8	79.5	63.7	55.9	...
B	5	100	100	100	100	100	100	100	100	100	99.2	100
	RT	100	100	100	100	100	97.0	100	89.1	100	84.4	94.0
	40	100	93.5	91.0	91.0	...	90.4	...	77.5	90.7	76.7	75.3
C	5	100	100	100	100	100	100	100	98.2	100	98.2	100
	RT	100	100	100	100	100	95.2	100	92.4	100	93.7	100
	40	100	100	100	100	100	87.0	100	91.3	100	89.7	81.0
D	5	100	98.1	100	100	100	99.5	100	90.3	...	...	100
	RT	100	98.5	100	96.8	100	95.4	100	89.5	...	...	93.6
	40	100	92.4	100	58.4	100	88.9	87.7	85.4	...	...	77.6
E	5	100	100	100	...	100	100	...	98.9	...	94.1	95.3
	RT	100	100	96.1	100	85.8	...	83.8	94.8	82.6	89.6	82.6
	40	100	99.0	88.5	95.5	85.8	...	82.0	86.3	82.3	78.9	76.5
F	5	...	Gelled	...	...	...	...	...	...	...	...	...
	RT	100	98.5	100	89.0	100	87.0	93.1	83.6	81.3	77.6	...
	40	100	88.5	100	74.0	90.2	64.8	85.3	62.5	65.2	54.2	...
G	5	...	100	...	Gelled	...	...	...	...	...	...	...
	RT	100	100	100	91.2	100	88.6	96.5	76.9	82.7	71.9	...
	40	99.6	99.7	94.3	81.4	89.6	75.9	78.2	64.3	74.8	54.8	...
I	5	100	100	96.8	100	92.7	100	92.6	92.5	92.7	...	88.9
	RT	86.7	91.0	72.3	89.5	57.9	87.0	48.3	80.5	...	...	36.5
	40	58.2	82.2	38.7	77.4	...	75.7	...	59.4	...	...	...
J	5	55.2	90.7	Ppt.[d]	...	...	...	...	...	...	...	...
	RT	29.5	85.5	...	...	...	84.2	...	....	...	...	...
	40	0.0	82.9	...	...	...	70.5	...	...	...	...	...
M	5	...	100	94.9	100	...	...	...	...	...	...	...
	RT	71.2	98.5	54.7	96.8	...	...	...	...	...	...	...
	40	26.6	77.0	...	73.5	...	...	...	...	...	...	...

[a] RT = Room temperature (25–30°). [b] Cyanocobalamin. [c] Ascorbic acid. [d] Precipitation occurred.

TABLE V.—SUMMARY OF COLOR CHANGES IN SAMPLES OF ASCORBIC ACID AND CYANOCOBALAMIN–ASCORBIC ACID SOLUTIONS IN PROGRESSIVE STAGES OF DECOMPOSITION

	Initial	1	2	3	4	5
Cyanocobalamin	Pink	Pink	Pink	Pink	Pink	Pink
Ascorbic acid	Colorless	Slight yellow	Yellow	Orange	Brownish orange	Brown
Cyanocobalamin–ascorbic acid	Pink	Peach	Yellowish orange	Orange	Brownish orange	Brown

ing glucose. A vehicle composed of equal parts of propylene glycol and glycerin afforded maximum stability conditions for a mixture of cyanocobalamin and ascorbic acid. There was about 80% retention of cyanocobalamin and 85% retention of ascorbic acid in this vehicle after six months' storage at 40°. There was no loss of cyanocobalamin and about 10% loss of ascorbic acid on storage for six months at room temperature.

It is suggested that the decomposition products of ascorbic acid play an important part in the decomposition of cyanocobalamin. Investigations are continuing on various phases of the problem, and it is expected that the results will be published.

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Blood and Bone Marrow Changes in Elderly Patients, with Special Reference to Folic Acid, Vitamin B_{12}, Iron, and Ascorbic Acid

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The prevalence of anaemia and the occasional unexpected finding of low serum folic acid and vitamin-B_{12} concentrations in aged inpatients prompted this investigation.

Anaemia, usually with normoblastic haemopoiesis, is common in the elderly (Bedford and Wollner, 1958; Lawson, 1960; Geill, 1962). Serum folic acid concentrations may be in the lower half of the range found in younger healthy people (Hansen and Nyström, 1961), while decreased serum folic acid levels in 40 out of 50 entrants to an old people's home, with increased excretion of formiminoglutamic acid in 31 of the 50, have been reported by Read *et al.* (1965). Reduced intake of food was thought to be the likely cause of folic acid deficiency in this group. Fifteen of a group of 50 apparently healthy aged subjects living at home also had serum folic acid concentrations below the range accepted as normal by Read *et al.* The serum vitamin-B_{12} concentration has been found to be reduced in old people (Mollin and Ross, 1952; Boger *et al.*, 1955; Chow *et al.*, 1956; Gaffney *et al.*, 1957; Cape and Shinton, 1961; Geill, 1962) though some authors could not confirm this (Killander, 1957). Droller and Dossett (1959) found that, although age itself had no effect on the serum vitamin-B_{12} concentration, patients with confusion or with senile dementia had significantly lower concentrations. The amount of ascorbic acid in the leucocytes of old people who had been living at home has been found to be abnormally low (Denson and Bowers, 1961).

A survey by the King Edward's Hospital Fund for London (1965) has shown progressive reduction in food intake with increasing age in healthy women living alone.

Patients Studied and Methods Used

One hundred consecutive patients newly admitted to the care of one of us (L. W.) at Cowley Road Hospital, Oxford, were studied. Patients who had received antibiotics, chemotherapy, or blood transfusion within two weeks before admission or folic acid, vitamin B_{12}, ascorbic acid, or vitamin supplements within one month before admission were excluded. Of the patients studied, 27 men and 73 women, the normal sex distribution for the hospital, 12 were aged 60–69, 49 were aged 70–79, and 39 were aged 80 and over (Table I).

All patients had a full clinical assessment, which was recorded in detail on a prescribed form. Dietary and social history was also recorded.

TABLE I.—*Distribution of Patients Aged 60 and Over According to Age and Sex*

Age	No. of Patients		
	Male	Female	Total
60–64	1	5	6
65–69	1	5	6
70–74	6	13	19
75–79	9	21	30
80–84	6	18	24
85–89	3	8	11
90 and over	1	3	4
	27	73	100

Haemoglobin concentration and blood counts were done on venous blood by standard methods.

Bone-marrow Examination.—Ninety-four patients submitted to bone-marrow examination. Bone-marrow films were stained by the May–Grünwald–Giemsa sequence and also for haemosiderin with a filtered mixture of 2% potassium ferrocyanide solution and 2% hydrochloric acid maintained at 56° C. for 20 minutes. The percentage of normoblasts containing haemosiderin (sideroblasts) was determined and the marrow was classified into four grades depending on this percentage (grade 1, 0–10%; grade 2, 11–20%; grade 3, 21–50%; grade 4, 51% and over). Values over 20% were considered to be normal, in keeping with the normal ranges of 16–69% (Lawrence, 1959), 24–81% (Douglas and Dacie, 1953), and 20–90% (Kaplan *et al.*, 1954).

Serum vitamin-B_{12} estimations were made by the method of Spray (1955), *Lactobacillus leichmannii* being used. The normal range by this method on 222 subjects studied during 1964–6 was 140–1,200 $\mu\mu$g./ml. of serum (Spray, unpublished observations). Only 168 of these subjects are included in this survey.

Serum folic acid estimations were made by the method of Spray (1964), *L. casei* being used. The normal range is 2.1–28 mμg./ml. of serum.

Blood ascorbic acid was measured by the method of Roe and Kuether (1943), using 2,4-dinitrophenylhydrazine.

Results

The patients showed clinical evidence of a wide range of disorders—57 had ischaemic cerebral disease, 31 had ischaemic heart disease, eight had malignant disease, eight had fractures of femur or humerus, two had frank bleeding from the gastrointestinal tract, two others had peptic ulceration, two had malabsorption, and four had rheumatoid arthritis.

Anaemia and Bone-marrow Results

Anaemia, defined as a haemoglobin concentration of 11.7 g./100 ml. or less, was present in 33 patients. Ten of these had a hypochromic anaemia with a mean corpuscular haemoglobin concentration (M.C.H.C.) of 30% or less and a diminished sideroblast count (grades 1 and 2) in the bone-marrow film. Six were anaemic with M.C.H.C. 30–31.9% and a diminished sideroblast count, and two had a normochromic anaemia with a diminished sideroblast count. Thirteen with a normochromic anaemia and two with a hypochromic anaemia had large numbers of sideroblasts in the bone marrow (grades 3 and 4).

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Sixty-seven patients were not anaemic on admission. In 5 the bone marrow was not examined, in 39 the marrow film showed decreased numbers of sideroblasts (grades 1 and 2), and in 24 sideroblasts were numerous.

Haemopoiesis was normoblastic in all cases except one, a woman with reticulosarcoma. Examination of the bone marrow showed a macronormoblastic form of haemopoiesis and the serum vitamin B_{12} was 310 $\mu\mu$g./ml. and serum folic acid 3.2 mμg./ml.

The bone marrow appeared to be of poor cellularity in 21 patients, but in only three of these was the haemoglobin concentration below 11.7 g./100 ml.

Serum Folic Acid Concentration (See Fig. 1)

The range obtained in 93 control subjects drawn from hospital staff and visitors was 2.1–28 mμg./ml. The mean of 79 control subjects aged 17–59 was 7.8 mμg., nine of whom had values between 2.1 and 3, and the mean of 14 control subjects aged 60 and over was 7.7.

The sera of 99 patients tested gave a range of 0.8–17 mμg./ml., with a mean of 4.7. Twenty patients had values between 2.1 and 3 mμg./ml. The difference between the means of the patients and of the younger control group was highly significant ($P<0.001$). The number of older subjects was insufficient for statistical analysis. There was no obvious difference in levels between the two control groups.

The patients with rheumatoid arthritis, malignant disease, and malabsorption all had levels in the normal range.

Serum Vitamin-B_{12} Concentration (See Fig. 2)

The range obtained in 168 control subjects drawn from hospital staff and visitors was 140–1,200 $\mu\mu$g./ml. The mean of 106 control subjects aged 10–59 years was 425 and of 62 control subjects aged 60 years and over was 429. Forty-one of the combined group had values below 300, with none below 140.

The sera of 98 patients gave a range of 110–880 $\mu\mu$g./ml., with a mean of 359.6. Fifty had concentrations below 300 and three were below 140. At concentrations of 120, 120, and 110, these levels were not as low as is usually found in untreated pernicious anaemia. The difference between the mean of the patients and the mean of each of the two control groups was highly significant ($P<0.01$).

There was no tendency in the control groups for the vitamin-B_{12} concentration to fall with age.

Organic Brain Disease

The mean serum folic acid concentration in our patients with evidence of organic brain disease was 3.9 mμg./ml., whereas that in patients without evidence of organic brain disease was 5.8 mμg./ml., a difference which is statistically significant ($P<0.001$). All patients with levels below 2.1 mμg./ml. had evidence of organic brain disease (Fig. 3). The mean serum vitamin-B_{12} concentration in the same group of patients with evidence of organic brain disease was 348 $\mu\mu$g./ml. and that of the group without such evidence was 376 $\mu\mu$g./ml., a difference which is not significant ($P>0.10$). Criteria of organic brain disease were the presence of abnormal neurological signs localized to the brain, all of the patients with these signs having recent or old cerebrovascular disease or, in two patients, persistent mental impairment with air-encephalograms suggesting cortical atrophy.

Blood Ascorbic Acid Concentration

Twenty patients had values below 0.2 mg./100 ml. Nine had values of 0.1 mg./100 ml. or less; all nine were admitted to hospital in the winter months.

TABLE II.—*Serum Folic Acid Concentrations in Patients Aged 60 and Over, According to Social State*

Living	Serum Folic Acid Concentrations (mμg./ml.)			
	< 2·1	2·1–3	> 3	Total
	No. of Patients			
Alone	2	2	15	19
With spouse	4	9	13	26
„ sister/brother	0	1	10	11
„ daughter/son	3	5	20	28
In welfare home	1	2	6	9
„ other accommodation	0	1	5	6
	10	20	69	99

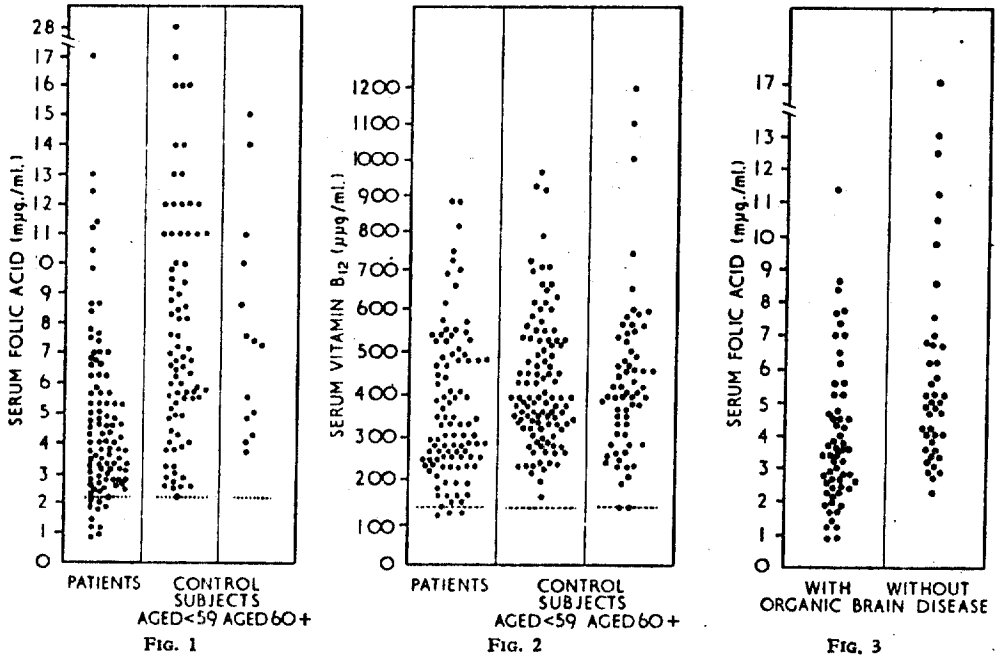

FIG. 1 FIG. 2 FIG. 3

FIG. 1.—Serum folic acid concentrations in patients aged 60 and over and in control subjects. FIG. 2.—Serum vitamin-B_{12} concentrations in patients aged 60 and over and in control subjects. FIG. 3.—Serum folic acid concentrations in patients aged 60 and over, with and without organic brain disease.

Values below 0.2 mg./100 ml. were commonly found in normal subjects taking 10 mg. or less of ascorbic acid a day (Medical Research Council, 1953), though values as low as 0.06 mg. were occasionally found with 20 mg. a day intake.

No statistically significant relation could be found between the following values: haemoglobin concentration, sideroblast count, and serum folic acid, serum vitamin-B_{12}, and blood ascorbic acid concentrations when taken in pairs. There was no correlation between serum folic acid concentration and social state (Table II).

Discussion

The spectrum of clinical disease encountered in this group of patients has been briefly described. It was not a group in which such haematological and vitamin assay findings would normally be expected.

A third of the patients were anaemic and a tenth had a frank iron-deficiency anaemia. In the whole group, evidence of iron deficiency as judged by reduced sideroblast counts was common. Normochromic anaemia was frequent, and poor cellularity of the bone-marrow sample was often found, but the two were not closely related.

Despite the prevalence of low serum folic acid concentrations and the general tendency for the serum vitamin-B_{12} concentrations to be reduced, there was no example of megaloblastic anaemia. The work of Read *et al.* (1965) indicated that old people with low serum folic acid concentrations were, in fact, deficient in folic acid, so that it is likely that the low levels in our patients were also indicative of folic acid deficiency. Presumably the deficiency had not become severe enough to cause recognizable megaloblastic anaemia in our group of patients.

Low blood ascorbic acid concentrations were common in the winter months despite the frequency with which old people ill at home were supplied with fruit juices rich in ascorbic acid. Any deficiency that there may have been was not sufficient to cause clinical scurvy,

Megaloblastic haemopoiesis due to folate deficiency in seven patients with peripheral neuritis and/or myelopathy was described by Grant *et al.* (1965). They thought that the most plausible explanation for the association was that neurological disease caused folate deficiency by interfering with appetite. This may have led to deficiency of other vitamins necessary for nerve function. Improvement brought about by folic acid in some cases may have been associated with improved appetite and subsequent improved intake of unknown factors.

Reduced serum folic acid levels and megaloblastic anaemia have been described in epileptics receiving anticonvulsant treatment. Malpas *et al.* (1966) confirmed that folic acid levels were often low in such patients, and they thought that anticonvulsant drugs which included barbiturates played a part along with nutritional deficiency. Fifteen of our patients had been taking barbiturates within one month before admission, but only three of these had subnormal serum folic acid levels.

Inquiry into the food intake of our patients led us to believe that this was often poor, but the difficulty of making an assessment on newly admitted gravely ill patients is very great and accurate information is lacking. In many cases there seemed to be no lack of availability of food; for instance, there was no greater tendency for low serum folic acid levels to occur among those living alone than among those living with younger relatives. The association with organic brain disease in our patients and of cord or peripheral nerve disease in the cases of Grant *et al.* (1965) suggested that physical disability or illness and disinterest in food were important factors. The part played by barbiturates is not clear, but interest in food might be blunted by treatment with them. In some cases sufficient suitable food was probably not available because of loss of interest, difficulty in swallowing, immobility, or poverty.

Reduced intake of food and malabsorption seem to be the two more likely causes of our findings and those of Read *et al.* (1965). We are puzzled by the lack of multiple deficiencies in the same patient on either score. Marked individual idiosyncrasy of diet might be responsible, but this seems unlikely. Malabsorption would tend to cause combined deficiencies of several factors in the same patient. In any case malabsorption on such a wide scale would be unlikely unless some process such as subclinical mesenteric ischaemia was much more widespread than is at present recognized.

It has recently been suggested that vitamin supplements would be of value to the elderly person living alone and that folic acid in doses of 25–50 μg. a day be given (Read *et al.*, 1965). Before such treatment is generally adopted it is our view that the problem should be more clearly defined. Our findings did not suggest that the old person at home alone was more likely to be deficient in these than a person living with relations. If apathy and disinterest and physical disability are more important than lack of availability of food then the provision of dietary supplements will be unlikely to solve the problem, for the supplement will be no more likely to be eaten than the food. Such supplements might even be dangerous in elderly people who could not be relied on not to exceed the recommended dose. In our view, if folic acid is given to the elderly, consideration should also be given to the need for providing parenteral vitamin-B_{12} treatment in addition.

Summary

Patients newly admitted to a geriatric department for various reasons often showed evidence of iron deficiency and low serum folic acid concentrations. Serum vitamin-B_{12} concentrations and blood ascorbic acid concentrations were also diminished. An association between organic brain disease and low serum folic acid was found. Possible reasons for the finding are discussed.

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Reactions of Cyanocobalamin and Aquocobalamin with Proteins.* (22268)

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(Introduced by Earl A. Hewitt.)

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It now appears probable that absorption, transport, storage and function of cobalamins are accomplished by vitamin-protein complex formation. Although there is ample evidence that cobalamins are bound by proteinaceous substances present in gastric and duodenal secretions, blood serum, liver tissue, and milk there is relatively little information concerning the nature of bonds involved in the binding reactions. Cooley and co-workers(1) postulated that cobalamin-protein reactions may occur via cobalichrome formation analogous to the formation of ammonia and histidine cobalichromes. Van der Zant and Underkofler(2) presented evidence that suggests participation of both cobalt and substituted benzimidazole of cobalamins in cobalamin-gastric mucosal extract reaction. Absorption spectrum of purified cyanocobalamin-gastric protein complex, reported by Wijmenga and co-workers(3), indicates that cyano-group may still be present in the complex. Gregory and Holdsworth(4) reported the isolation of a similar cobalamin-protein complex from sow's milk and presented evidence indicating that the cyano-group is not displaced in the formation of this complex, and that the complex will not accept a second cyano-group. The latter authors made an extensive study of this binding reaction and concluded that tyrosyl residues of proteins but not sulfhydryl or amino groups, may participate. This investigation presents further

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FIG. 1. Dialysis cells and rotation apparatus.

evidence concerning mode of linkage involved in cobalamin-protein reactions.

Dialysis method. Obvious inconsistencies in bacterial inhibition(5,6) and bacterial uptake(7) methods for differentiating free and bound cobalamins led to adoption of a dialytic procedure and radioisotope assay. Initial trials using dialysis bags and dialyzing to equilibrium or exhaustion, indicated that these methods were time-consuming and mechanically awkward. By standardizing the volume, membrane area and agitation a short-term or partial dialysis method was devised. Three identical Plexiglas dialysis cells were used. Each cell half contained a milled-out channel of about 30 ml capacity and a filling hole. The membrane, Visking dialysis tubing slit along one edge, was stretched between the two sections, separating and sealing the channels when cell halves were bolted together. Cells and rotation apparatus (3 r.p.m.) are shown in Fig. 1. In use, 25 ml of buffered protein-Co^{60}-labeled cobalamin mixture were dialyzed against equal volume of the same buffer for 4 hours in the dark at room temperature. Both solutions were then subjected to radio assay using a G-M liquid counting detector. Concentration of protein was adjusted to bind about half of the labeled cobalamin present. Using dialysis rate of free cobalamin, as determined by omitting protein from one of the 3 cells, the amount of cobalamin rendered non-dialyzable by a given amount of protein was calculated. Radioactive cobalamins were derived from a Co^{60}-labeled preparation (225 μc/mg cobalamin) obtained from Merck and Co. on AEC allocation. Cyanocobalamin (vit. B_{12}) was prepared by treatment of an aqueous solution of this preparation with an excess of NaCN followed by nitrogen aeration in the dark at pH 6.5. Aquocobalamin (vit. B_{12b}) was prepared by simultaneous illumination and nitrogen aeration of an aqueous solution of this preparation at pH 5.5. All subsequent operations using these preparations were carried out under conditions of minimum light. Dialysis rate of vit. B_{12} followed Fick's diffusion law, that is, directly proportional to concentration differential across membrane at constant temperature and membrane area, and was unaffected by buffer type or concentration, or pH over the range 2 to 10. Dialysis rate of vit. B_{12b} was less straightforward, being complicated by adsorption by the cellophane membrane, and perhaps dimerization, the effect being more prominent in media of low ionic strength. By using 0.2 M phosphate buffer reproducible dialysis of vit. B_{12b} was obtained at observed rate approaching that of vit. B_{12}.

Results. Using the partial dialysis method, it was found that many proteins bind vit. B_{12b} to a much greater extent than vit. B_{12}, as indicated in Table I. Although not hitherto

TABLE I. Binding of Vit. B_{12} and B_{12b} by Proteins.

Protein preparation*	Bound cobalamin, mμg/mg N†		
	B_{12}, pH 6.6	B_{12b}, pH 6.6	B_{12b}, pH 4.0
Whole hemolysed blood	0	3.8	—
Hemolysed red cells	0	3.0	—
Plasma	0	5.0	.7
Plasma, heat denatured‡	0	5.8	—
Albumin (Armour)	0	3.0	.7
Lysozyme, 2× crystallized	0	0	—
Lysozyme, alkali denatured§	0	3.7	—
Ovalbumin	0	3.7	—
Trypsin, 2× crystallized	0	2.5	.5
Pepsin, " "	0	5.1	—
Gastric mucosal extract	340	340	320

* Preparations contained 1 mg N/ml (0.013 mg N/ml for gastric mucosal extract) and 7.6 mμg cobalamin/ml in 0.2 M phosphate buffer. Blood preparations were of bovine origin.

† Sensitivity ca. 0.2 mμg cobalamin/mg N.

‡ Heated at 100°C for 5 min.

§ Incubated in 0.1 N NaOH at 37°C for 2 hr.

reported, this difference in extent of reaction between the two forms of vitamin can be expected as manifestation of cobalichrome formation. It was found that denaturation increased the vit. B_{12b} binding capacity of plasma and lysozyme. The binding capacity of 3 preparations was markedly decreased in acid media. Unreported spectrophotometric studies(8) indicated that vit. B_{12b}-histidine reaction is similarly depressed in acid media, as expected, and that histidine is the only amino acid giving evidence of cobalichrome formation in neutral solution. Similar compounds such as histamine, imidazole, carnosine, histidyl histidine and pyridine were found to form cobalichromes with vit. B_{12b}.

However, it cannot be inferred that benzimidazole is the only possible vit. B_{12b} binding site, for it was observed(8) that substances such as cellophane, filter paper, heparin, and nucleic acids (DNA and RNA) rendered nondialyzable considerable amounts of vit. B_{12b}. These substances did not bind measurable amounts of vit. B_{12}. The vit. B_{12b} bound by these substances and by the protein preparations was released by treatment with excess of cyanide in neutral solution.

The binding of as much as 5 mμg of vit. B_{12b}/mg of N by plasma is of interest for the physiological level is in the order of only 0.02 mμg of cobalamin/mg of N. Pitney, Beard and Van Loon have reported(9) that naturally occurring cobalamins are present in alpha and beta globulin components and that these components may also bind additional amounts of cobalamins, presumably vit. B_{12}, to a maximum of about 0.05 mμg/mg of N.

The position of bound labeled vit. B_{12} and B_{12b} in bovine serum was determined by subjecting to paper electrophoresis exhaustively dialyzed serum-cobalamin mixtures, and measuring radioactivity of the separate protein components(10). Serum used in this study was found to retain 4.3 mμg vit. B_{12b} or 0.092 mμg vit. B_{12}/mg of N, after exhaustive dialysis. Results presented in Table II indicate that all fractions of serum participated in binding of vit. B_{12b}, whereas only beta and gamma globulin components bound vit. B_{12}.

TABLE II. Position of Bound Co^{60}-Cobalamins in Bovine Serum Fractions Obtained by Paper Electrophoresis.

Preparation	Percent of activity			
	Albumin	α-globulins	β-globulins	γ-globulins
Serum + Co^{60}-B_{12b}	7	28	20	45
" " " -B_{12}	0	0	82	18

An entirely different situation was encountered in studying reaction of cobalamins with swine gastric mucosal extract. This extract consisted of water soluble, 50% alcohol insoluble, nondialyzable components of Ventriculin (Parke-Davis). As shown in Tables I and III, this preparation bound rather large and equal amounts of vit. B_{12} or B_{12b}, and binding of one form of vitamin was essentially blocked by prior excess of the other.

The effect of pH and excess cyanide on

TABLE III. Binding of Vit. B_{12} and B_{12b} by the Gastric Extract (GE).

Components*	Bound labeled cobalamin,† mμg/mg N
GE + Co^{60}-B_{12}	350
GE + excess B_{12b} + Co^{60}-B_{12}‡	0
GE + Co^{60} B_{12b}	350
GE + excess B_{12} + Co^{60}-B_{12b}‡	15

* Preparations contained 0.013 mg N/ml, 7.6 mμg labeled cobalamin/ml, and 2 μg nonlabeled cobalamin/ml, in 0.2 M phosphate buffer of pH 6.6.
† Sensitivity ca. 10 mμg cobalamin/mg N.
‡ Gastric extract and nonlabeled cobalamin allowed to react before adding labeled cobalamin.

TABLE IV. Effect of Hydrogen Ion Concentration and Excess Cyanide on Binding of Vitamin B_{12} by Gastric Extract.

pH*	Bound vit. B_{12}, mμg/mg N	
	No CN^-	Excess CN^-
2	320	—
4	320	—
6	330	—
6.6	340	340
8	320	—
10	330	250
10	340	250†
10	330	200‡
11	220	80
12	90	0

* Preparations contained 0.013 mg N/ml and 7.6 mμg Co^{60}-B_{12}/ml in 0.2 M phosphate buffer.
† Gastric extract and B_{12} allowed to react before adding cyanide. In all others, cyanide and B_{12} mixed before adding gastric extract.
‡ Contained 0.4 mg NaCN per ml. All others contained 0.1 mg NaCN/ml.

TABLE V. Fate of Cyano Group of Vit. B_{12} in the Vit. B_{12}-Gastric Extract Reaction.

Treatment	Gastric extract	H_2O control
CN^- removed initially by N_2 aeration		
In dark, mμg	86	--
Illuminated, mμg	15	--
Idem	8	5
B_{12} added in dark, μg	4.5	4.5
(CN^- equivalent, mμg)	(87)	(87)
CN^- recovered by N_2 aeration		
In dark, mμg	14	12
Illuminated, mμg	79	83
B_{12} bound,* μg	3.4	--
(CN^- equivalent, mμg)	(66)	--

* Determined by the partial dialysis method.

binding of vit. B_{12} by this extract is shown in Table IV. The reaction was essentially unaffected by pH changes from pH 2 to 10 but was inhibited in more basic solution. Excess cyanide had no effect in neutral solution, but exerted a progressive inhibition above pH 10. The order of addition of excess cyanide and labeled vit. B_{12} to gastric extract did not affect the magnitude of inhibition. Excess cyanide did not affect the dialysis rate of free cobalamin. These data suggest that cobalamin is bound to this protein through the position sought by the second cyano-group.

That the cyano-group of vit. B_{12} is not displaced by the binding reaction is indicated by data presented in Table V. The cyanide method of Boxer and Rickards(11) was used. Gastric mucosal extract was first freed of cyanide by nitrogen aeration at pH 5. Labeled vit. B_{12} was then added and cyanide again determined, first after aeration in the dark, then while being illuminated. The amount of vit. B_{12} which had actually been bound by gastric extract was subsequently determined by partial dialysis. It appears that the first cyano-group is not displaced by the binding reaction, but remains in a photolabile position, presumably still with the cobalamin structure.

The effect of several treatments on vit. B_{12}-gastric mucosal extract reaction, as studied by partial dialysis, is shown in Table VI. The reaction was not affected by type or concentration of buffer. The effect of heat was similar to that reported by others using bacterial assay methods(2,12,13) and indicates that the complex is as heat-labile as the unreacted binding substance at pH 6.6. The report that cobalt-benzimidazole mixtures inhibit the reaction(2) could not be confirmed by this method. The suggestion that protein sulfhydryl groups may be involved in binding was shown to be unlikely, for the reaction was unaffected by excess para-chloromercuribenzoate. A cobalt porphyrin, reported to promote growth of vit. B_{12}-requiring microorganisms(14) was found to have no marked effect on the reaction.

Discussion. The marked reactivity of vit. B_{12b} suggests that suitable precautions should be taken against photoconversion when attempting to study vit. B_{12}-protein reactions. For example, heparin and nucleic acids were found to bind vit. B_{12b}, and not vit. B_{12} as had been reported(15). The binding or adsorption of vit. B_{12b}, by substances such as cellophane and filter paper point up difficulties involved in ascribing a physiological function to substances showing vit. B_{12b}-binding activity. Data presented in Tables I and

TABLE VI. Effect of Various Treatments on Binding of Vit. B_{12} by the Gastric Extract.

Treatment		Bound vit. B_{12}, mμg/mg N
Effect of buffer at pH 6.6		
No buffer		350
.1 M phosphate		320
.2 "		330
.4 "		330
.2 M acetate		340
Heating the gastric extract		
60°C, 1 hr		330
100°C, "		180
Heating the B_{12}-gastric complex		
60°C, 1 hr		330
100°C, "		180
Reagents	**Molar excess***	
$CoCl_2$	2×10^6	320
Benzimidazole	3×10^6	330
" , $CoCl_2$	" , 2×10^6	320
" , " , heat†	" , "	270
p-Chloromercuribenzoate	1×10^4	340
Cobalt-hematoporphyrin	1×10^5	310

* Relative to original B_{12} concentration, 7.6 mμg per ml or 5.6×10^{-9} M.

† Heated at 100°C for 10 min. prior to addition of B_{12}. Gastric extract alone given same heat treatment bound 290 mμg B_{12}/mg N.

II suggest that binding of vit. B_{12b} by proteins, possibly via a cobalichrome linkage with histidine residues, is a rather general reaction involving little structural specificity of the proteins involved, and may therefore be of little physiological significance.

The limited study of the cobalamin-gastric mucosal extract reaction reported here is in general agreement with the more extensive study of Gregory and Holdsworth(4) who used ultrafiltration to study binding of vit. B_{12} by an isolated milk protein. However, there appears to be some discrepancy concerning the effect of excess cyanide on the reaction or reaction product. Gregory and Holdsworth observed no changes in absorption spectrum of cyanocobalamin-milk protein complex in presence of excess cyanide at pH values as high as pH 11. The data presented here indicate that dicyanocobalamin, in presence of excess cyanide, does not react completely with gastric extract, that excess cyanide dissociates pre-formed cyanocobalamin-gastric extract complex, and that these reactions are dependent upon cyanide concentration and pH. These results may represent a difference in nature of the binding reaction by the 2 preparations, or a difference in molar excess of cyanide used in the two situations. Although there have been reports (16,17) that cobalamin-peptide complexes are split by cyanide in neutral solution, the possibility exists that these are cobalichrome type structures and thus more susceptible to cyanolysis.

Summary. 1. A short-term dialysis procedure has been developed for studying cobalamin-protein reactions. 2. Aquocobalamin (vit. B_{12b}) was much more reactive than cyanocobalamin, being bound in rather large amounts by several protein preparations of diverse origin. In several instances binding was increased by denaturation and decreased in acid media. It is suggested that this type of binding may occur via cobalichrome formation. 3. A gastric mucosal extract bound large and equal amounts of aquocobalamin and cyanocobalamin, apparently at the same binding site. Excess cyanide has no effect on the cyanocobalamin-gastric extract reaction in neutral media, but exhibits an inhibiting effect at high pH levels. In this reaction the cyano-group of cyanocobalamin is not released, but remains in a photolabile position. It is suggested that the cobalt atom participates in the reaction and that it is the position occupied by the substituted benzimidazole moiety that is involved.

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INFANTILE GENETIC AGRANULOCYTOSIS ASSOCIATED WITH CHANGES IN SERUM VITAMIN B_{12} BINDING PROTEINS

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Neutropenias occurring during the neonatal period have been recently reviewed by Kauder & Mauer (16). One of the most serious forms of such neutropenias, infantile genetic agranulocytosis, was first described by Kostmann in 1956 (18). In this condition there is a severe defect in the production of neutrophil granulocytes, the affected children presenting with bacterial infections during the first few weeks of life and often dying in infancy.

There are two main vitamin B_{12} binding proteins in serum (11) an α-globulin, transcobalamin I (TCI) and a β-globulin, transcobalamin II (TCII). In normal adult serum nearly all the endogenous vitamin B_{12} is bound to TCI and only a small fraction is carried on TCII. TCII however, has a considerable capacity to bind added B_{12} whereas the binding capacity of TCI is almost completely saturated. Early studies on serum showed that both the endogenous B_{12} level and the unsaturated vitamin B_{12} binding capacity were elevated in conditions of granulocyte proliferation (2, 24). These findings suggested that TCI was a breakdown product of granulocytes and the presence of B_{12} binding protein in these cells was demonstrated by Mollin & Ross (24). Meyer et al. (23) subsequently showed that mature neutrophil granulocytes contained the highest concentration of this protein. Radioisotope studies in vitro have confirmed that a TCI-like protein can be synthesised in granulocytes (7, 29) but recent evidence suggests that it may be secreted into the plasma from the intact cells rather than occur as a breakdown product (5, 7). In addition to TCI and TCII a third vitamin B_{12} binding protein has been described in cord and neonatal serum by Kumento et al. (20) and Kumento (19). This appears to be an α-globulin of similar molecular weight to TCI but by contrast does not carry endogenous B_{12} and thus its B_{12} binding capacity is unsaturated. It probably disappears from the plasma within a few weeks of birth.

In this paper the clinical course of a case of infantile genetic agranulocytosis is described. In view of the severe defect in granulocyte production serum B_{12} binding proteins were analysed. Electron microscopy of bone marrow cells was undertaken in an attempt to define more precisely the morphologic changes present in the granulocyte cells in this disease.

Clinical summary

D. B. was born on 15th February, 1969 following a full-term gestation and normal pregnancy and delivery. The birth weight was 3.4 kg. He progressed normally until the 6th day when a small area of erythema was observed around the umbilicus. Apart from a rectal temperature of 38.5°C (101°F) the physical examination was normal. Over the next few days the erythema spread and was accompanied by induration of the surrounding skin extending to 5 cm in diameter (Fig. 1). The umbilicus remained dry. These signs presented a most unusual appearance, quite unlike that found even in severe purulent infections of the umbilicus

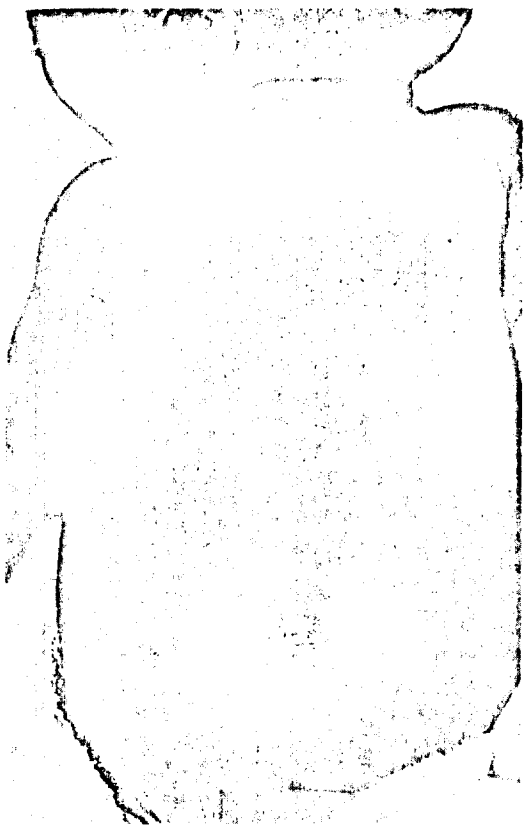

Fig. 1. Case D. B. at 21 days of age.

At 3 weeks an erythematous indurated area about 0.5 cm in diameter was noted in the left groin. Pus was not found in either of these two lesions and no granulocytes were seen in the slight serous discharge. A swab from the umbilicus grew a haemolytic streptococcus. Despite continuous antibiotic therapy (see Fig. 2) the umbilical sepsis and fever continued until death. At 5 weeks of age the abdomen suddenly became distended due to both hepatic enlargement and ascites and at this time the serum bilirubin was 5.3 mg/100 ml and the serum glutamate oxalate transaminase 335 units. Repeated paracenteses during the remainder of his life removed about 2 litres of clear bile stained fluid. The baby gradually became cachectic and died on 6th May at the age of 11 1/2 weeks.

Family history

Both parents are healthy and unrelated. The father was aged 40 and the mother 29 at the time of conception. The only abnormality detected in either parent was a slight eosinophilia of 450 to 700/mm³. In the father this eosinophilia was present on three of the four occasions his blood was tested. In the mother the eosinophilia was noted on two of four occasions. There are two normal sibs, one older and the other younger than D. B.

Investigations

A nearly complete agranulocytosis persisted from the first blood counts when 6 days old to the time of death (Fig. 2). A very occasional neutrophil was seen and in 27 differentials (2 700 cells counted) only 3 neutrophils were noted. This was associated with a low grade eosinophilia (up to 1 400/mm³) and a marked monocytosis (up to 5 000/mm³). The lymphocyte count was usually between 4 000–7 000/mm³. The haemoglobin level was persistently low and a transfusion was required when 26 days old. The platelet count was initially normal. These findings are summarised in Fig. 2. No maternal leucocyte agglutinins were present. Immunoglobulin levels, stool tryptic activity and urinary excretion of amino acids were all normal.

The bone marrow was examined on two occasions and was of increased cellularity. No neutrophil granulocyte precursors beyond the promyelocyte stage were present. The marrow differential is shown in Table 1. A number of the promyelocytes were morphologically abnormal with prominent cytoplasmic vacuolation (Fig. 3). The marrow revealed a marked lymphocytosis with increases in both eosinophils and plasma cells. Small clear vacuoles were present in some of the plasma cells.

Electron microscopy of the bone marrow showed that many of the eosinophilic granules were abnormal. The granules were ring-shaped with pale central areas (Fig. 4), and the normal crystal structure was not seen. The vacuoles in the plasma cells were also demonstrated (Fig. 5). Much granular material was present in macrophages (Fig. 6). In several areas of the marrow, collagen fibres were present (Fig. 7).

Direct chromosome analysis of bone marrow cells failed to reveal analysable mitoses on two occasions. Stimulation of peripheral blood lymphocytes by phytohaemagglutinin gave normal chromosome findings.

Serum B_{12} was measured by *E. gracilis* assay (1). The B_{12} level was 208 pg/ml when the serum was heated prior to assay and 105 pg/ml without heat treatment. B_{12} bound to TCI only becomes available to microbiological assay organisms after destruction of TCI by boiling. B_{12} attached to the β globulin binder (now known as TCII) can be assayed without boiling (15). The unsaturated B_{12} binding capacity (10) was 445 pg/ml. Separation of B_{12} binding proteins by gel filtration on Sephadex G. 200 was undertaken using the method of Hom et al. (13). The results of the separation are shown in Fig. 8. No definite binding of added radioactive B_{12} (^{57}Co cyanocobalamin) was seen in the position occupied by the TCI and foetal binders (peak at tube no. 70) which are not separable by gel filtration (19).

Post-mortem Findings

The body was that of a wasted infant. There was much bile stained fluid in the peritoneum with many

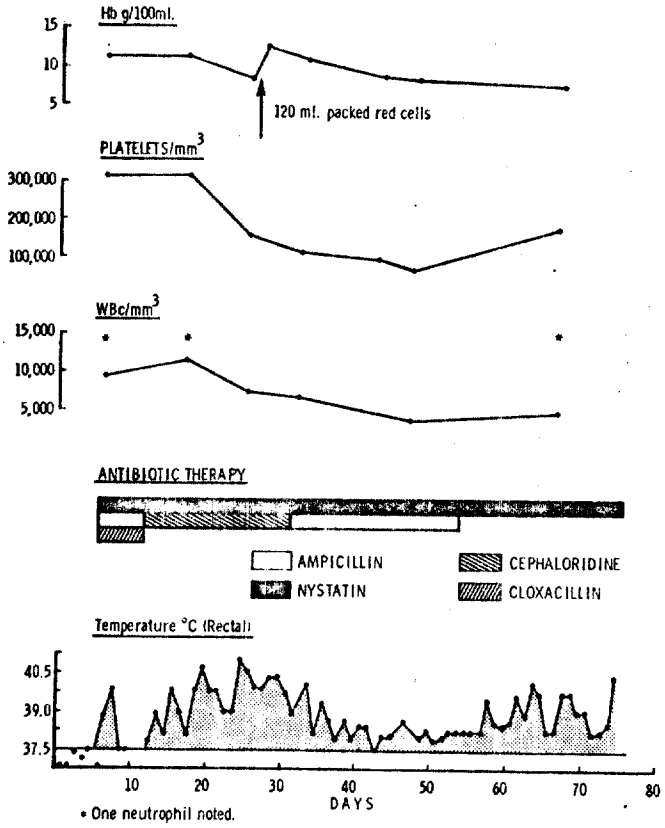

Fig. 2. Clinical course of case D. B.

fibrinous adhesions. The cells in the peritoneal exudate were almost entirely mononuclear cells and eosinophils but a very occasional neutrophil granulocyte was seen. The liver was enlarged (340 g) and showed extensive centrilobular parenchymal necrosis, fibrosis and calcification. No thrombi were present in the main hepatic veins, but there was extensive occlusion of small hepatic venous radicles and this presumably caused the liver cell necrosis. Recent thrombi were also present in the small pulmonary arteries, epididymis, thyroid, retroperitoneum, and renal veins. The spleen (31 g) and left inguinal lymphnodes were enlarged. The other lymphnodes were normal in size, and the thymus was noted to be atrophic. The retroperitoneal lymphnodes exhibited severe lymphoid cell depletion, but the inguinal nodes and the spleen were normal except for a small area of necrosis in one inguinal node. No definite bacteria could be identified with any of the tissues examined.

COMMENT

Infantile genetic agranulocytosis was first described by Kostmann in 1956 (18). In his series skin and respiratory infections during the first

Table 1. *Marrow differential count (1000 cells) expressed as a percentage*

	Case D. B.	Control normal values[a]
Myeloblast	1.5	0–2
Promyelocyte	1.5	0–4
Myelocyte	None	1.5–13.5
Metamyelocyte	None	4–31
Segmented neutrophil	None	2–27
Eosinophil myelocyte	3.5	0–1
Eosinophil metamyelocyte	3.5	0–1
Segmented eosinophil	1.5	0–1
Segmented basophil	0.2	0
Proerythroblast	None	0–0.5
Early normoblast	1.0	0–1.5
Intermediate normoblast	2.5	0–3.5
Late normoblast	4.0	1–15
Plasma cell	0.5	0
Lymphoblast	2.5	0.5–3
Prolymphocyte	4.0	—
Lymphocyte	69.8	27–73
Reticulum cell	4.0	0

[a] Gairdner et al. (8).

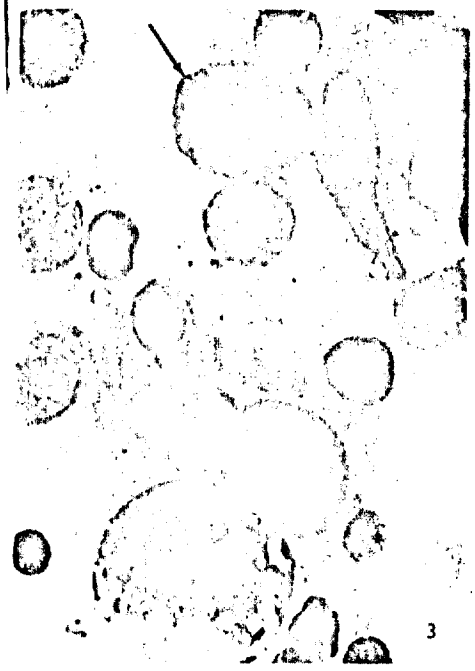

Fig. 3. Marked vacuolisation of promyelocytes. The finer vacuolisation present in plasma cells is indicated by the arrow. × 1 500.

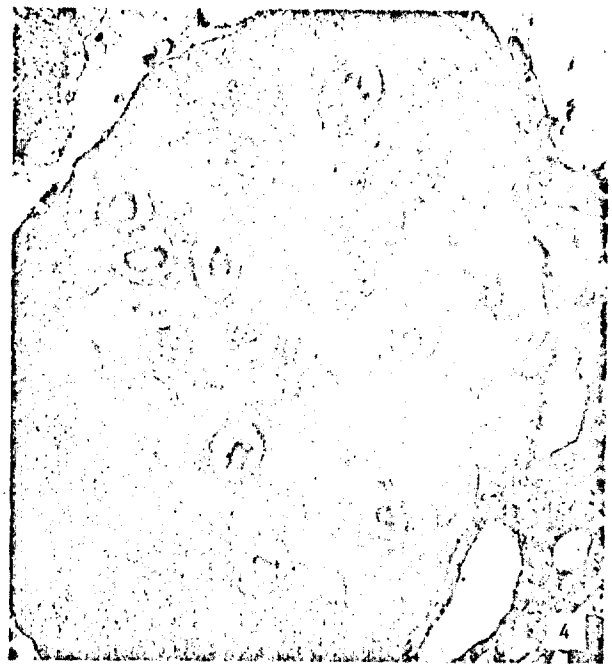

Fig. 4. Eosinophil showing abnormal granules. × 15 000.

weeks or months of life were the presenting features and in most cases the affected children died during the first two or three years of life. Examination of blood and marrow in several of these children revealed a severe neutropenia with an "arrest" of neutrophil

Fig. 5. Plasma cell showing endoplasmic reticulum and inclusions. × 15 000.

Fig. 6. Macrophage containing granular material. × 10 000.

Fig. 7. Collagen fibres in bone marrow. × 15 000.

granulocyte production in the bone marrow at the myelocyte–promyelocyte stage. A high incidence of consanguinity was present in Kostmann's cases and he concluded that the disease was probably caused by an autosomal recessive genetic defect. Subsequent reports have been reviewed by Gilman et al. (9) who point out that the mechanism of inheritance was not always similar to that described by Kostmann, since many occur as single cases with no other affected family members. The severity of the impairment of granulocyte production is variable, the milder defects carrying a better prognosis. Several cases reports (3, 9) include children still alive in the second decade of life, although in one instance death from leukaemia has been noted in these older children (9). The present case illustrates several of the findings described in Kostmann's monograph. Skin sepsis was the initial feature and this appeared during the first week of life. There was selective involvement of neutrophil granulocytes; apart from a slight reduction in the haemoglobin level the other cells derived from the bone marrow were normal or increased during the first few weeks of life. A normochromic normocytic anaemia and mild but transient thrombocytopenia then developed. It seems likely that vascular obstruction to the small tributaries of the hepatic vein caused the sudden onset of hepatic enlargment with ascites. It is possible that this and the terminal intravascular thrombosis were consequential to the umbilical sepsis and peritonitis. No macroscopic "abscesses" were present at the time of death and this is an unusual feature, but the appearances at post mortem were probably modified by the continuous antibiotic therapy and the severity of the granulocyte production defect.

Several morphological abnormalities of blood and bone marrow cells were described by Kostmann. The presence of eosinophilia and monocytosis is a common finding. In the marrow there is usually a marked lymphocytosis. The granulocyte precursors are frequently abnormal and Kostmann recorded that promyelocytes and myelocytes were often atypical with lobed and vacuolated nuclei. Vacuolation may also affect the cytoplasm and this is illustrated in Fig. 3. This vacuolation is similar to that seen in chloramphenicol toxicity (27)

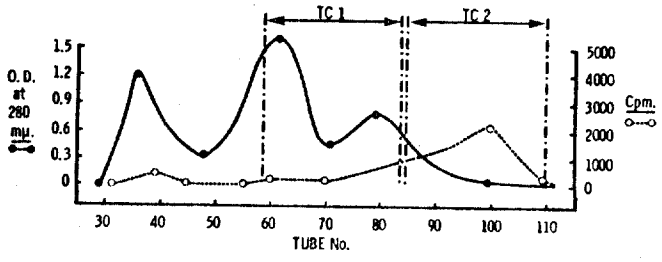

Fig. 8. Separation of vitamin B_{12} binding proteins.

after ingestion of alcohol (25) riboflavin deficiency (21) and phenylalanine deficiency (6). No information was obtained from the electron microscopy studies concerning the nature of this granulocyte vacuolisation. Abnormal granules were present in some but not all eosinophils and none of the eosinophil granules contained the characteristic crystals.

Chromosome findings have been reported in only one previous case described as infantile genetic agranulocytosis (22). There must be some doubt over the diagnosis in this case since the 37 day old infant had a severe pancytopenia and was thus quite unlike the cases described by Kostmann. Direct bone marrow chromosome study revealed many abnormalities but examination of peripheral blood lymphocyte chromosomes was not reported. In our case direct marrow chromosome analysis failed, but chromosome analysis of peripheral blood lymphocytes was normal. Schroeder & Kurth (28) grouped Kostmann's Agranulocytosis together with Fanconi's Anaemia as conditions in which a high incidence of chromosome breakage is known to occur. This seems premature since the evidence for this is based on the above report (22), and furthermore in our case lymphocyte chromosomes were normal in contrast to the many abnormalities found in Fanconi's Anaemia.

In normal adult sera TCI is largely saturated with endogenous B_{12} and on addition of radioactive B_{12} *in vitro*, 50–90% of the radioactivity binds to TCII (12). However, in neonatal sera as little as 10% of the added radioactivity may bind to TCII (19) the rest being bound to the TCI and/or foetal binders. The serum from case D. B. had a low unsaturated vitamin B_{12} binding capacity and all the added radioactive B_{12} was bound to TCII (Fig. 7) thus indicating the absence of any foetal binder and the absence of any unsaturated TCI. The serum B_{12} level of case D. B. (208 pg/ml) was below the normal range for a child of that age (17) and about 50% of the serum B_{12} could be assayed without boiling the serum. Thus the amount of TCI present in the serum of D. B. has a total vitamin B_{12} binding capacity of only about 10[illegible] pg/ml.

The results in the present case show that there is probably a total absence of foetal binder and a marked reduction of TCI in the serum. This is consistent with the current hypothesis that serum TCI is largely derived from mature neutrophil granulocytes and furthermore provides indirect evidence that the foetal binder may be derived from the same cells. The small amount of saturated TCI present may have been derived from monocytes, as these cells have been shown to contain a B_{12} binder indistinguishable from that in granulocytes (4). It is unlikely to have come from eosinophils (23). The presence of a relatively large proportion of serum vitamin B_{12} which could be assayed without boiling may be a result of liver cell necrosis which can lead to release of free B_{12} or TCII bound B_{12} into the serum (14, 26).

SUMMARY

A case resembling infantile genetic agranulocytosis is reported. A nearly complete absence of neutrophil granulocytes in the peripheral blood was associated with an arrest of neutrophil granulocyte formation at the promyelocyte stage in the bone marrow. Electron microscopy of bone marrow revealed abnormalities of both eosinophils and plasma cells. Studies of vitamin B_{12} binding proteins showed a marked reduction of TCI and/or foetal binders.

The significance of these findings is discussed. They provide indirect evidence that TCI and/or foetal B_{12} binding proteins are derived from developing and mature neutrophil granulocytes. Further studies of B_{12} binding proteins and of bone marrow morphology should be undertaken in congenital defects of granulocyte production.

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THE LIVER, RADIATION AND LEUKEMIA*

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ARTICLES and editorials appearing in the literature are emphasizing with increasing frequency the relationship between radiation and leukemia.[5-9] Statistical studies of the incidence of leukemia in radiologists, Hiroshima victims, and recipients of radiation therapy have revealed increased incidence of leukemia following roentgen-ray exposure.[4] This phenomenon, late in developing, makes causal relationship difficult to assess. Relatively little experimental work and very little documentation of the possible mechanisms involved have been reported.

There are at present two principal approaches to the study of the mechanisms involved in leukemia. The first and perhaps most popular is the report by a number of observers that leukemia can be transmitted by an agent similar to a filtrable virus. The second approach is the attempt to demonstrate in the leukemias biochemical abnormalities which might be responsible for the disease. Neither of these approaches has been entirely successful to date. There are many and obvious blank spaces in our knowledge of the mechanisms of human leukemia. A discussion of these mechanisms is still highly theoretical, but evidence is now accumulating which makes a theoretical discussion of etiologic relationships timely.

In 1954 we reported the finding of excessive amounts of protein-bound vitamin B_{12} in the blood serum of patients with myelocytic and monocytic leukemia, both acute and chronic.[1-3] This phenomenon is not present in lymphatic leukemia. In innumerable determinations in patients with both acute and chronic myelocytic and monocytic leukemia, the finding of protein-bound B_{12} values of from two to fifteen times the values of normal sera has been consistent, and numerous other observers have now verified this finding. Elevations of serum B_{12} have also been reported in cirrhosis of the liver, and occasionally in myelofibrosis and polycythemia vera. The serum B_{12} elevations in cirrhosis of the liver present certain differences, consisting chiefly of saturation of the protein binding and a normal clearance of cobalt 60 labeled B_{12}. The elevations that are reported occasionally in myelofibrosis and polycythemia reflect on the nature of these diseases, since both have many features in common with myelocytic leukemia.

These elevated serum B_{12} values in leukemia are protein-bound B_{12} values as measured by the *Euglena gracillis* technique of microbiologic assay. The *Euglena gracillis* is an organism which is capable of differentiating between protein-bound and free B_{12}. A typical sampling of normal bound B_{12} values as compared with nonlymphatic leukemias is shown in Figure 1.

The protein binding of B_{12} in the serum as studied by microbiologic assay with *Euglena gracillis* and paper electrophoresis was reported by us in 1954.[11] In the normal serum almost all of the bound B_{12} can be recovered in the cut strips which exhibit alpha globulin mobility. A typical measurement of this phenomenon superimposed on a Tiselus curve is shown in Figure 2. Repeated studies of this phenomenon by microbiologic assay have been consistent. Attempts to locate the binding with Co^{60} labeled B_{12} have not been successful in our hands.

Study of the location of the bound B_{12} by similar techniques in the nonlymphatic leukemic sera has also revealed the rather specific alpha globulin location of the bind-

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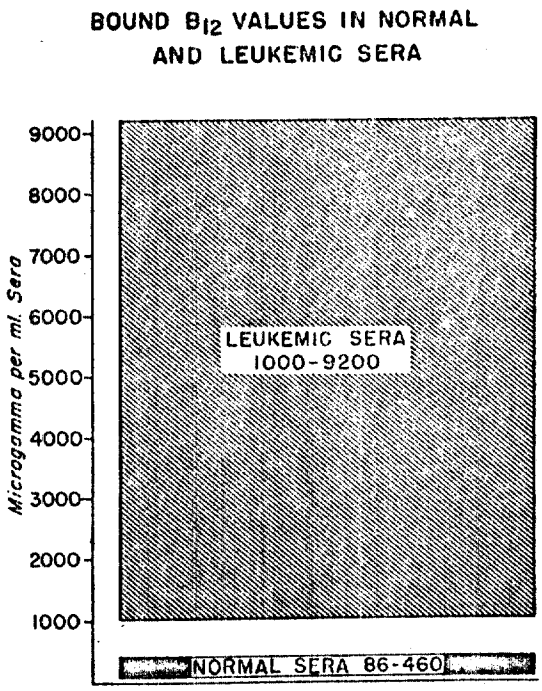


Fig. 1.

ing. This is illustrated in Figure 3. While the location of the bound B_{12} has been in the fractions of alpha globulin mobility, repeated electrophoretic study of the leukemic sera has failed to disclose an excessive amount of alpha globulin, and we feel that the difference in binding is qualitative rather then quantitative.

Studies of this abnormal binding have been interesting. *In vitro* studies of normal sera show, by bioassay techniques, a rather limited capacity for the sera to bind B_{12}. An increase approximately twice the normal values seems to be maximum. Studies of the leukemic sera, on the other hand, reveal an almost unlimited capacity to bind B_{12}. This is demonstrated in Figure 4. *In vivo* studies also reveal a very abnormal capacity to bind B_{12}, as shown in Figure 5.

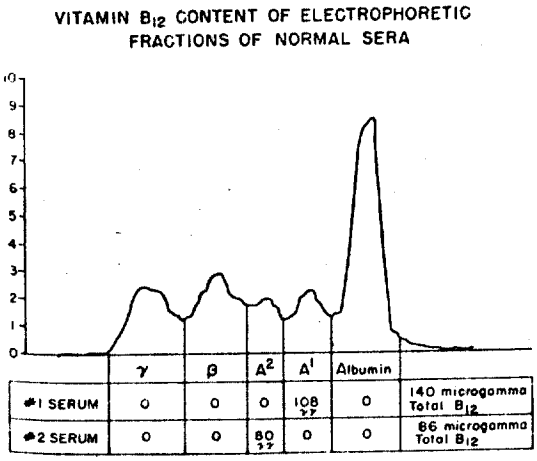


Fig. 2.

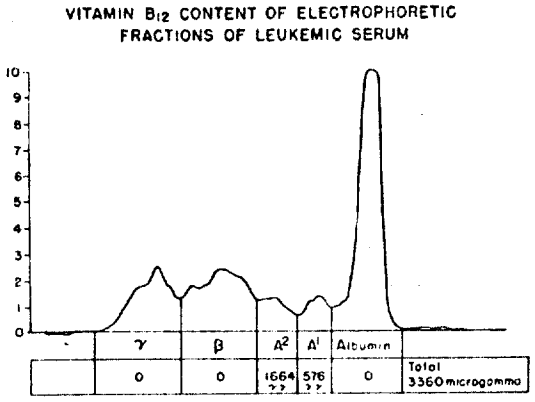


Fig. 3.

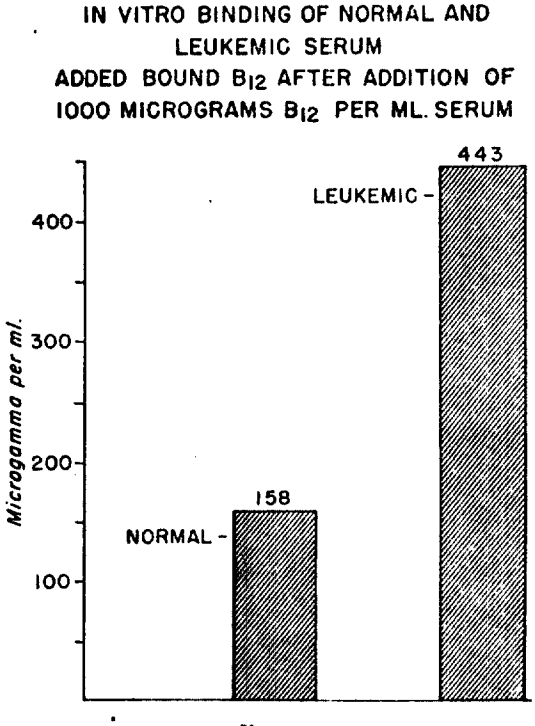
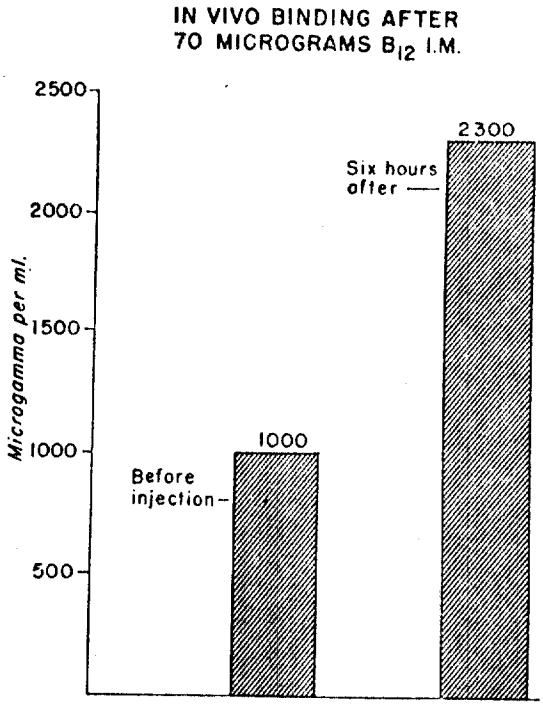


Fig. 4.



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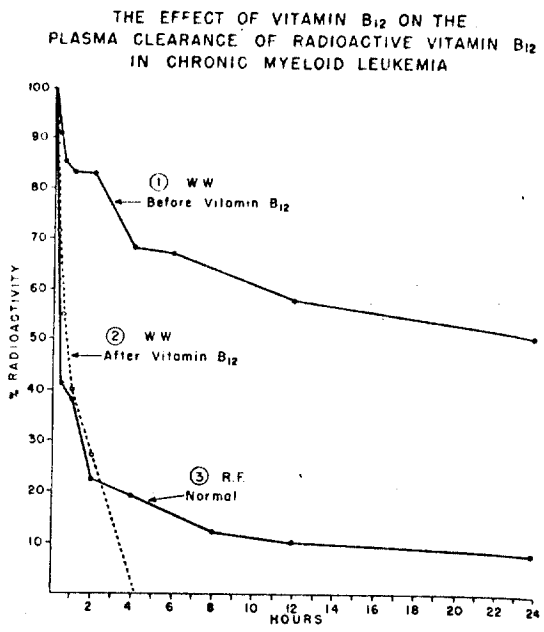

Fig. 6. Reproduced by permission of authors, editor and Grune & Stratton, Inc., New York.[12]

Studies of the plasma clearance of Co^{60} labeled B_{12} in the normal and in the leukemic likewise reveal marked differences. In the leukemic individual, if relatively enormous doses of nonradioactive B_{12} are given parenterally, the binding capacity of the serum can be saturated and the plasma clearance returned to normal (Fig. 6).

There is some evidence to indicate that the protein bound B_{12} is the biologically active form of the vitamin. Most biochemists are agreed that vitamin B_{12} is an essential factor in the synthesis of nucleoproteins. Whether excessive amounts of protein bound B_{12} can result in excessive nucleoproteins in the leukemias as has been reported is rather controversial. We have approached this question indirectly in two ways: (1) We have attempted to lower artificially the high bound B_{12} values in patients with leukemia. Figure 7 shows the result of such an attempt by using exchange transfusions. The high B_{12} levels could not be returned to normal, and the moderate lowering of the B_{12} levels was quite transient. In spite of this, there was some lowering of the peripheral blood levels but no change in the bone marrow or clinical picture; (2) we have attempted to block nucleoprotein synthesis by the use of an antagonist—6 mercaptopurine, and have observed the effect on the serum B_{12} levels and on the

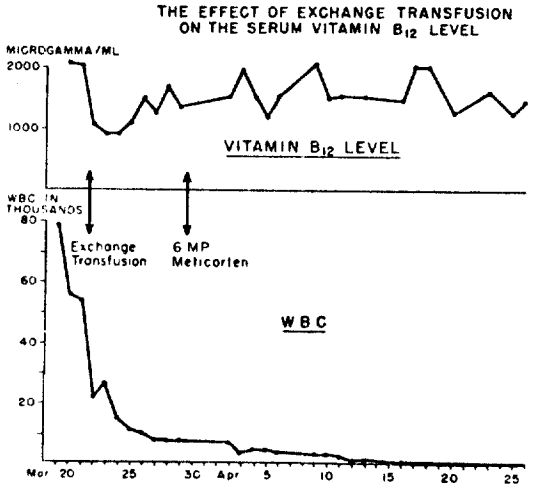

Fig. 7. Reproduced by permission of authors, editor and Grune & Stratton, Inc., New York.[12]

clinical picture of the leukemia. Figure 8 shows these results. While the patient may go into a good clinical remission, there is no lowering of the serum B_{12} levels. Remissions induced by myeleran, a cytotoxic agent, are, on the other hand, accompanied by a reduction in the values of bound B_{12} in the serum. This is illustrated in Figure 9.

While these results are preliminary and inconclusive, they are suggestive of the fact that the elevated bound B_{12} values may be the actual mechanisms whereby the abnormal amounts of nucleoproteins and the wild production of immature cells by the bone marrow are initiated.

The fundamental mechanism involved in these nonlymphatic leukemic patients is a qualitative abnormality of the alpha globulin insofar as B_{12} binding is concerned. This abnormal alpha globulin is able to combine with B_{12} in excess, either from the liver or from minute increases over a long period

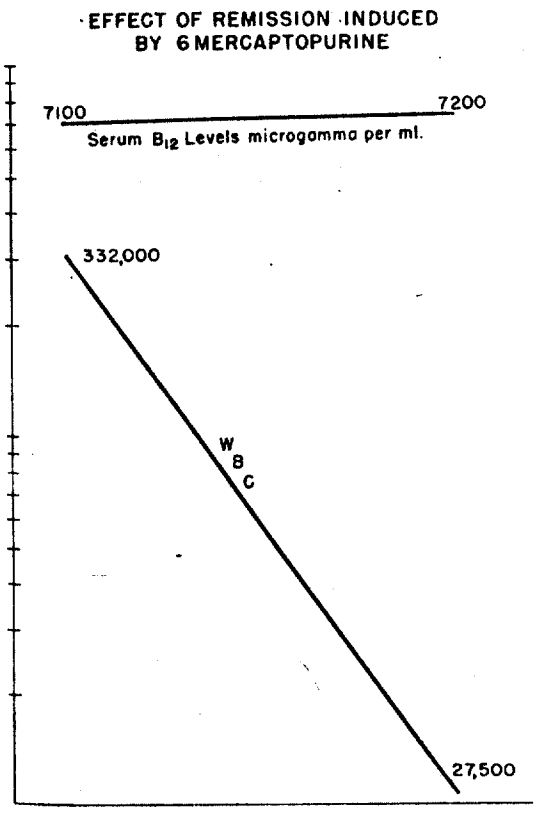

Fig. 8.

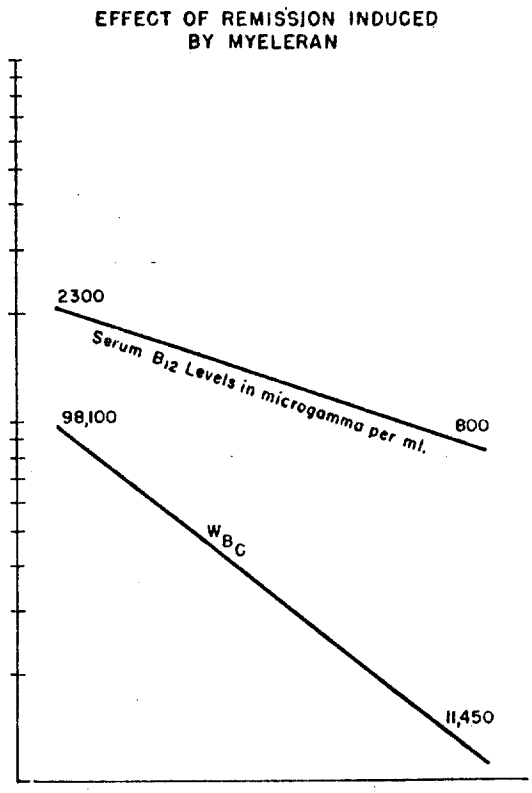

Fig. 9.

from the intestinal tract. Present techniques do not demonstrate increased absorption from the intestinal tract, but they are not refined enough to demonstrate slight increases of absorption.

The source of the abnormal alpha globulin is hepatic. Miller and Bale,[10] in a series of ingenious experiments, have shown that almost all of the alpha globulin is hepatic in origin. Rachmilewitz *et al.* have also demonstrated a transient increase in alpha globulin bound B_{12} following acute chemical injury to the liver. Thus we can postulate that chronic liver injury, from irradiation, from chemical poisoning, or from infection such as a virus, may result in an abnormal alpha globulin, which is capable of binding abnormal amounts of vitamin B_{12}.

Hepatic effects of radiation have been described by numerous investigators and that the liver is particularly sensitive to irradiation is well known.[7] To date no ex-

periments have been done to prove that irradiation over a period of years may result in abnormal alpha globulin. In view of the collateral evidence reported, it would seem, however, that such a concept would not be too illogical. The difficulties of experimental documentation are obvious. In all reports on radiation and leukemia, the remoteness of the development of the leukemia following irradiation is emphasized. Acute experiments thus being useless, only time and new techniques will aid in proving the correctness of this concept.

SUMMARY

It is postulated, that the following relationship exists between irradiation and leukemia: chronic radiation effects on the liver result in the production of an alpha globulin which has a peculiar affinity for B_{12}. This protein gradually binds excessive amounts of vitamin B_{12} from the liver, or from an intestinal tract containing normal amounts of the vitamin. This excessive bound B_{12} is biologically active, resulting in abnormal production of nucleoproteins and excessive formation of immature cells, leading to the clinical picture we call leukemia. The process may well be reversible at some stage of its development.

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Effect of Vitamin B_{12} on Liver Regeneration after Partial Hepatectomy

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Previous investigators have shown. that after carbon tetrachloride injury, there is a decrease of the hepatic vitamin B_{12} content [17, 18, 34, 38] concomitant with an increase of the serum vitamin B_{12} content [2, 17, 18, 34, 41]. It has also been established that administration of vitamin B_{12} prior to or simultaneously with the carbon tetrachloride intoxication prevents the increase in lipids [26] and bromosulfophthalein retention [26] and the depletion of ribonucleic acid [17, 26], protein [17], and phospholipids [17]. In a previous paper [4] we have demonstrated that vitamin B_{12}, also when given after the intoxication, has a beneficial effect upon the liver, evident from a more rapid restoration of the hepatic content of glutamic pyruvic transaminase (GPT).

Stein, O., Stein, Y., Aronovitch, Grossowicz, and Rachmilewitz [34] have shown, that after partial hepatectomy there is a decrease to about fifty per cent of the vitamin B_{12} content in the liver on the second postoperative day. The restoration of the hepatic B^{12} content to its preoperative values lags behind the growth of the regenerating organ, although the hepatic uptake of radioactive vitamin B_{12} in the liver was significantly increased as compared to sham-operated and to normal controls. This finding gives rise to the question, if administration of vitamin B_{12} to hepatectomized animals stimulates the liver healing as was the case with the regeneration after carbon tetrachloride injury. This has previously been investigated by *Ferrari and Brondon* [10], who could find no effect upon the liver weight, liver protein content, or number of mitoses. The doses of the vitamin were, however, very small and the experiments were terminated already on the fourth postoperative day. *Schweigert, Scheid, and Downing* [32] and *Wong and Schweigert* [38] observed lower growth rate and liver content of ribonucleic acid and desoxyribonucleic acid four days after partial hepatectomy in animals fed a vitamin B_{12}-deficient diet as

compared to rats supplied with the vitamin. *Maros, Hadnagy, Seress-Sturm, Csiky, and Kovács* [23], after injection of moderate doses of the vitamin, could demonstrate a slight but significant stimulation upon the wet liver weight restoration seven days but not three days after partial hepatectomy. *Damm, Ralli, Gershberg, and Laken* [9] studied the effect of vitamin B_{12} upon the liver regeneration in hepatectomized rats reared on a low protein-high fat diet 42 days before the operation and up to 14 days postoperatively. Instead of a continued increase of the fat content, as seen in the controls reared on the same diet, a marked decrease of the liver lipids was evident in the B_{12} treated animals. A decrease of the same magnitude was obtained also if the diet was changed to a moderate protein diet. No attempt was made to influence the lipid content in the latter animals by administration of vitamin B_{12}.

In the present investigation we wanted to study the effect of vitamin B_{12} after partial hepatectomy in animals whose liver lipid content was normal before the operation, to see if also the fatty metamorphosis that normally occurs after partial hepatectomy could be prevented by administration of the vitamin. As we have previously observed a beneficial effect of the vitamin upon the restoration of the hepatic GPT in liver healing [4] the content of this enzyme in the liver was also studied.

Methods and Material

The material is presented in Table I. Male Sprague-Dawley rats, 4 to 5 months old, were used. They were reared on an adequate laboratory diet. To avoid differences in food intake no food or water was given on the day of operation. The operations were performed under general ether anesthesia. At the operation we used a technique of our own [5] whereby we make a subtotal resection of the median, left lateral, right anterior and right posterior lobes, leaving only the two small caudate lobes intact. The animals received an intramuscular injection of 75 000 IU DBED-penicillin and 75 000 IU procaine penicillin. One group of the animals was treated with 50 µg of vitamin B_{12} supplied by ASTRA, Sweden, injected subcutaneously at the operation and at daily intervals during the postoperative period till the day before sacrifice. The animals were killed by a blow on the neck at different intervals from 1 day to 28 days after the partial hepatectomy. The weight of the animal as well as the dry and wet liver weight was

TABLE I

Number of Animals, Initial Body Weight, and Liver Remnant in the Studied Groups

Day at which the animals were subsequently sacrificed	Number of animals		Initial body weight, g Mean (σx̄)		Liver remnant in % of total preoperative liver Mean (σx̄)	
	Controls	B_{12}-treated	Controls	B_{12}-treated	Controls	B_{12}-treated
2nd postoperative	8	6	343 (8.0)	403 (14.2)	34.9 (1.18)	32.2 (1.09)
4th postoperative	15	6	345 (7.2)	316 (11.7)	36.5 (0.91)	34.3 (1.33)
8th postoperative	9	5	361 (9.5)	345 (8.4)	36.0 (1.06)	38.7 (1.36)
28th postoperative	8	4	350 (9.5)	355 (6.5)	32.4 (0.98)	39.4 (1.50)

determined. Estimations of the content of glutamic pyruvic transaminase (GPT) in the liver tissue was performed using *Ordell's* modification [24] of *Karmen's* method [16]. For these analyses we used commercial reagents, manufactured by *Kabi*, Sweden. The preparation of liver homogenates was in accordance with the method of *Schmidt et al*, [31]. One unit on enzyme activity is equivalent to a change in optical density of 0.001 per ml per minute at wave length 340 mμ. A detailed description of these procedures is given in another paper [3]. For fat determination we used the method described by *Shipley et al.* [33].

Results

Changes in body and liver weights (Table II).

With the exception of the second postoperative day, when the body weight loss is significantly less in the vitamin B_{12} treated animals, no statistically significant changes between the two groups are apparent. Nor are there any statistically significant changes in the restoration of the wet as well as of the dry liver weight. Because of the very great difference in fat content between the two groups 4 days after the operation (see below) we have also computed the restoration of the dry, fatfree liver weight. This is significantly ($0.01 > p > 0.001$) greater in the B_{12} treated group on the fourth postoperative day indicating a more rapid true regeneration in these animals.

TABLE II

Results of Body and Liver Weight Determinations

Days after operation	Body weight loss in % Mean ($\sigma\bar{x}$)		Wet liver weight in % of initial wet liver weight Mean ($\sigma\bar{x}$)		Dry liver weight in % of initial dry liver weight Mean ($\sigma\bar{x}$)		Dry fatfree liver weight in % of calculated preop. fatfree liver weight Mean ($\sigma\bar{x}$)	
	Controls	B_{12}-treated	Controls	B_{12}-treated	Controls	B_{12}-treated	Controls	B_{12}-treated
0			34.6 (0.76)	35.3 (0.71)				
2	10.4 (0.98)	7.3 (0.48)	46.3 (2.80)	42.5 (3.88)	48.2 (3.85)	45.8 (3.58)	34.5 (5.75)	29.5 (4.28)
4	8.8 (1.27)	10.7 (2.25)	56.5 (2.43)	57.5 (3.87)	45.4 (2.44)	51.5 (4.85)	32.8 (3.07)	48.5 (5.10)
8	8.2 (0.89)	7.5 (2.12)	70.7 (2.14)	62.5 (2.45)	66.7 (3.63)	60.5 (2.45)	60.1 (4.12)	66.5 (2.45)
28	+3.4 (1.32)	+2.5 (2.08)	85.5 (2.56)	76.3 (2.40)	82.5 (5.00)	72.5 (5.41)	84.5 (4.08)	72.0 (4.76)

Changes in total lipid content (Table III)

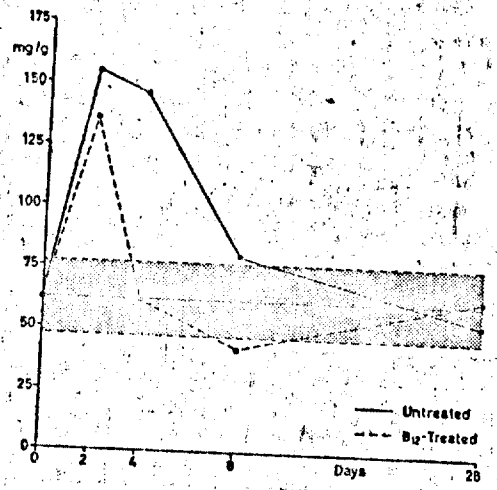
There is no significant difference between the two groups in fatty infiltration on the second postoperative day. The decrease of the liver fat occurs, however, much more rapidly in the treated group. On the fourth postoperative day, the total lipid content in the treated group is fully normalized, whereas no significant decrease has yet occurred in the controls. Also on the eighth postoperative day, the total lipid content in the treated animals is significantly lower than in the controls, although none of the groups differs from the normal value. No differences are apparent one month after the operation.

Changes in GPT content (Table IV, V)

There is a much more rapid decrease of the GPT content after the partial hepatectomy in the control group. The difference between the two groups 2 days after the operation is statistically significant. The restoration of the enzyme content occurs with the same rate in the two groups, but the content is higher in the B_{12} treated animals. Although there is no statistically significant difference between the two groups on the fourth, eighth or 28th day after the operation, analysis of variance on the values of the animals killed on these days, i.e. dur-

TABLE III

Total Lipid Content in mg/g Wet Liver Tissue after Partial Hepatectomy



Days after hepect.	*Untreated* No. of anim.	Mean	$\pm 2 \cdot \sigma\bar{x}$	*B_{12}-Treated* No. of anim.	Mean	$\pm 2 \cdot \sigma\bar{x}$
0	16	62.0	47.2–76.8			
2	4	157.0	119.2–194.8	4	134.5	81.9–187.1
4	6	145.8	101.6–180.0	11	62.7	49.9–75.5
8	9	80.1	67.1–93.1	7	43.1	35.1–51.9
28	8	53.1	45.9–60.3	6	64.5	51.9–77.1

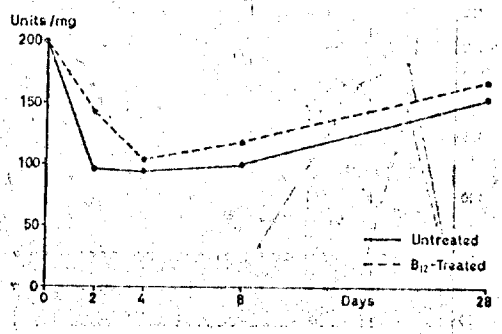
ing the period of restoration, shows that the values for the B_{12} treated group are significantly different than those for the controls (Table V). Possible effects due to interaction are non-significant.

Discussion

The low hepatic content and the high serum concentration of vitamin B_{12} in various conditions of liver damage have mostly been explained as resulting from a release of the vitamin from damaged liver cells [2, 17, 37]. The question if there is also a decreased uptake of the

TABLE IV

Content of GPT (Units/mg Dry Liver Tissue) at Different Intervals after Partial Hepatectomy



Days after hepect.	Untreated No. of anim.	Mean	(± 2·σx)	x	B_{12}-Treated No. of anim.	Mean	(± 2·σx)	σx̄
0	11	201	158–244	21.3				
2	8	98	84–112	6.9	7	146	124–168	11.1
4	7	93	69–117	12.0	4	105	71–39	17.1
8	9	101	81–121	10.2	6	120	91–149	14.5
28	6	155	131–179	12.0	6	166	140–192	13.0

vitamin by the liver has been differently answered by different investigators. Thus, *Glass, Boyd, and Ebin* [12], in a clinical study of various human liver diseases, found deficient uptake of vitamin B_{12} in 11 out of 36 cases. *Stevenson and Beard* [37], however, in another clinical study, obtained results indicating normal hepatic uptake of the vitamin. It is probable that the capacity of the liver to absorb the vitamin is differently affected by different causes of liver damage. The experiments performed by *Stein, O., Stein, Y., Aronovitch, Grossowicz, and Rachmilewitz* [34] convincingly demonstrate that after partial hepatectomy there is an increased ability of the liver to take up the vitamin. It is also evident that the vitamin is essential for the regeneration, as previous investigators have shown a slower growth rate of the liver remnant in animals fed a B_{12} deficient diet. The question, if it is possible to further stimulate the regeneration in animals fed a usual, adequate laboratory diet has, however, not been satisfactorily an-

TABLE V

Statistical Analyses of Data Obtained from GPT Determinations 4 to 28 Days after Partial Hepatectomy

Source of variation	Degrees of freedom	Sum of squares	Variance (S^2)	Variance ratio (F)
Treatments	1	15 477	$S_1^2 = 15\,477$	$\frac{S_1^2}{S_6^2} = 10.4$ (significant) $F_{0.05=4.11}$
Time after operation	2	16 086	$S_2^2 = 8\,043$	$\frac{S_2^2}{S_6^2} = 5.43$ (significant) $F_{0.05=3.27}$
Interaction	2	3 142	$S_3^2 = 1\,571$	$\frac{S_3^2}{S_4^2} = 1{,}03$ (not signif.) $F_{0.05=<3.27}$
Error	34	50 318	$S_4^2 = 1\,480$	
Error, interaction included	36	53 460	$S_5^2 = 1\,485$	
Total	39	85 023	$S_6^2 = 2\,180$	

swered. The experiments of *Ferrari and Brondon* [10] were already terminated on the fourth postoperative day, and very small doses of the vitamin were used. *Maros, Hadnagy, Seress-Sturm, Csiky, and Kovács* [23] used moderate doses, and made observations up to the seventh postoperative day, but as only the wet liver weight was determined, we do not know if the greater weight of the livers in the treated animals was due to a real increase of functioning liver parenchyma. The prerequisites for a beneficial effect of increased supply of vitamin B_{12} seem, however, to be present, as the animals studied by *Stein, O., Stein, Y., Aronovitch, Grossowicz, and Rachmilewitz* [34] and reared on a stock diet showed a lag of increse of the vitamin B_{12} content behind the growth of the regenerating organ in spite of an increased uptake of the vitamin.

Several effects have been ascribed to vitamin B_{12}. The lipotropic effect, which has been observed under such different conditions as after low protein-low fat and low protein-high fat diet [12, 13, 22], after carbontetrachloride intoxication [17, 20, 21, 26] and after pancreatectomy [25], has been differently explained. It now seems to be generally accepted that vitamin B_{12} acts upon the synthesis of methionine. In 1950, *Juke, Staktad, and Broquist* [15] demonstrated that vitamin B_{12} deficient chicks were not able to transform homocystine

into methionine. In the same year *Dubnoff* [8] could show that a greater synthesis of methionine from homocystine occurred in liver slices in the presence of vitamin B_{12}. It has also been shown that after administration of vitamin B_{12} plus folic acid supply of "labil-methyl-compounds" in the diet is unnecessary, provided that the diet contains homocystine [29, 35]. The methyl-groups, available from methionine, facilitate the synthesis of choline, which is a building stone of lecithin. Lecithin in its turn makes the transport of fat from the liver to the depots possible. Methyl-groups can also be synthesized directly from formic acid if vitamin B_{12} and folic acid is present.

The facts, shortly summarized above, thus indicate that the main effect of vitamin B_{12} upon the lipid metabolism is a transport-promoting one, leading to an increased transport of neutral fat from the liver to the depots. This explanation seems to be valid also for the results obtained in the present investigation, as the vitamin prominently accelerated the decrease of lipid content from the second postoperative day. It is of interest in this connection that we have previously observed a sharp increase of the phospholipid content in the liver [7] from the second to the fourth postoperative day. After this day, there was a decrease of the phospholipid content till the sixth day. It seems probable that the increase of the phospholipids is due to a mobilization of fat in the liver. In view of the fact, mentioned above, that vitamin B_{12} indirectly increases the formation of lecithin, it seems probable that an earlier and/or more pronounced rise of the phospholipid content occurs in animals treated with vitamin B_{12}, making a more rapid utilization of the neutral fat possible. It is worth pointing out that administration of the vitamin did not prevent the fatty metamorphosis as very high values were obtained on the second postoperative day. In a previous investigation [6] we have studied the effect of testosterone upon the liver healing after partial hepatectomy. In the animals injected with this hormone the total lipid content was within normal limits 2 days after the operation. We cannot explain this difference, we just want to point out the discrepancy in results obtained after the two treatments, indicating that the lipotropic mechanism of testosterone is somewhat different from that of vitamin B_{12}.

We have previously observed a stimulating effect upon the restoration of the hepatic content of GPT after carbon tetrachloride injury [4]. A similar effect was obtained after partial hepatectomy. Thus, it seems plausible that vitamin B_{12} has a stimulating effect also upon

the protein metabolism. According to *Koch-Weser and Popper* [20] vitamin B_{12}, through its facilitating effect upon the formation of ribonucleic acids, influences the protein formation, which in turn may control the enzyme regeneration in liver damage. This facilitating effect upon the nucleic acid synthesis seems to be well established. In 1949 *Stern, Taylor, and Russell* [36] demonstrated that rats given a diet deficient in vitamin B_{12} showed no liver basophilia in contrast to those receiving the vitamin. In 1951 *Alexander and Hacklar* [1] demonstrated that rats fed a vitamin B_{12} deficient diet had significantly less nucleic acid in the liver than those receiving the vitamin. This has been confirmed by other investigators [27, 28, 32, 39]. Because of a supposed relation [19, 36, 40] between vitamin B_{12} and ribonucleic acid synthesis, *Popper, Koch-Weser, and Szanto* [26] had already in 1949 tested the effect of vitamin B_{12} upon the centrizonal depletion of ribonucleic acid that occurs after carbon tetrachloride intoxication. The beneficial effect of the vitamin after this kind of liver damage was lately confirmed by *Kasbekar, Lavate, Rege, and Sreenivasan* [17]. It has also been demonstrated that the restoration of the hepatic content of ribonucleic acid after partial hepatectomy is impaired in animals given a diet deficient in vitamin B_{12} [32, 39].

The fact that we, in spite of the otherwise beneficial effect of the vitamin, could demonstrate no effect upon the restoration of the liver weight must be considered in view of the finding [30] that the liver weight is higher in animals deficient in vitamin B_{12} than in animals supplied with the vitamin. This seems to be due to the significant increase of the mean cytoplasmic volume in vitamin B_{12} deficiency, demonstrated by *Rasch, Swift, and Schweigert* [27]. We know from the experiments of *Stein, O., Stein, Y., Aronovitch, Grossowicz, and Rachmilewitz* [34] that there is a relative deficiency of vitamin B_{12} in the liver during the regeneration after partial hepatectomy.

Summary

The effect of administation of vitamin B_{12} to rats subjected to $^2/_3$ partial hepatectomy was studied 2, 4, 8, and 28 days after the operation. No effect was obtained upon the restoration of the liver weight. Four days after the partial hepatectomy the total lipid content was already normalized in the treated animals, being still markedly elevated in the controls. A statistically significant stimulation of the restoration of liver glutamic pyruvic transaminase was observed.

Zusammenfassung

Der Einfluß von Vitamin B_{12} auf Ratten mit $^2/_3$Partialresektion der Leber wurde untersucht, und zwar 2, 4, 8 und 28 Tage nach der Operation. Eine Wirkung auf das Lebergewicht war nicht festzustellen. 4 Tage nach der Partialhepatektomie war der Totalfettgehalt normalisiert, während er bei den Kontrollen erheblich gesteigert war. Statistisch signifikant war die Wiederherstellung der Glutamin-Brenztraubensäure-Transaminase.

Résumé

Des rats auxquels on avait réséqué les $^2/_3$ du foie furent traités par 50 µg de vitamine B_{12} chaque jour dès l'opération, et sacrifiés 2, 4, 8, et 28 jours plus tard.

L'effet fut nul sur la récupération pondérale du foie. Mais 4 jours après l'hépatectomie partielle, la teneur du foie en lipides totaux était revenue à la normale; et la vitamine B_{12} stimule, de façon statistiquement significative, la restauration de la transaminase glutamino-pyruvirique du foie.

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Vitamin-B_{12} Activity in Red Cells

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It has been suggested that the concentration of vitamin B_{12} in the serum may not necessarily reflect the true level of the vitamin in the tissues in some patients (Mollin and Ross, 1957; Spray, 1962). Booth and Spray (1960) showed that in rats after total gastrectomy the level of vitamin B_{12} in the serum became subnormal before that in the liver. Sobotka, Baker and Ziffer (1960) found that while the plasma of normal subjects contained more vitamin B_{12} than the red cells, the red cells of untreated patients with pernicious anaemia usually contained more vitamin B_{12} than the plasma. Thus when deficiency of vitamin B_{12} develops in man, the concentration of the vitamin in red cells may fall more slowly than that in the plasma, as does the level of vitamin B_{12} in the livers of rats after total gastrectomy. Hence it seems possible that a more exact picture of the amount of vitamin B_{12} in the tissues may be obtained by measuring vitamin-B_{12} activity in red cells.

In this paper the levels of vitamin B_{12} in the packed red cells of control subjects are reported. Results have also been obtained from patients suffering from or believed to be developing deficiency of vitamin B_{12}, from patients who had had a gastrectomy, and from patients with hypochromic anaemia, leukaemia and polycythaemia. These results are compared with those from the control subjects.

MATERIALS AND METHODS

Subjects Studied

Blood was obtained from 50 control subjects, 26 men and 24 women aged from 17 to 85 years, who were either hospital staff or haematologically normal patients. The patients studied included 12 with untreated pernicious anaemia, eight with 'latent pernicious anaemia' (Callender and Spray, 1962), five who had been treated for hypochromic anaemia, 10 who had had a partial or total gastrectomy, 11 with chronic myeloid leukaemia, four with chronic lymphatic leukaemia, four with acute leukaemia of various types, and 10 with polycythaemia vera.

Assay of Vitamin-B_{12} Activity in Red Cells

Blood (10–15 ml.) was taken by venepuncture, heparinized, and centrifuged at 2800 rev./min. for 30 minutes. Samples (2 ml.) of plasma and packed red cells were removed and stored at −15° C. in plugged 20 ml. graduated test tubes until they were assayed. The red cells were not washed, because experiments showed that there was no significant difference between the concentrations of vitamin B_{12} in washed and unwashed samples from four subjects (Table I). In a fifth subject, who had chronic myeloid leukaemia, washing reduced the level of vitamin B_{12} by less than 50 per cent even though the level in the plasma was 17,000 μμg./ml.

Vitamin-B_{12} activity was assayed by the method of Spray (1955) using *Lactobacillus leichmannii* as test organism. Extracts of red cells were prepared for assay by adding 2 ml. of 0.4 M-acetate buffer, pH 5.23, and 0.4 ml. of 0.1 per cent (w/v) NaCN solution to 2 ml. of packed red cells, and diluting the mixture to 20 ml. with distilled water. The samples were

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then autoclaved, cooled and centrifuged in the same way as described for serum (Spray, 1955). The resulting extracts, which were clear and pale pink in colour, were added to the basal medium without further treatment. It was shown that the colour of the extracts did not affect the opacity readings, and that the heparin which was added to the blood did not contain any measurable vitamin-B_{12} activity. Vitamin-B_{12} activity in plasma was assayed by the same method as used for serum (Spray, 1955).

TABLE I

THE EFFECT OF TWO WASHINGS WITH 0.9% NaCl ON THE VITAMIN-B_{12} ACTIVITY OF PACKED RED CELLS

Subject No. and diagnosis	*Vitamin-B_{12} activity (µµg./ml.)*		
	Unwashed red cells	*Washed red cells*	*Plasma*
1. Control	135	140	580
2. Control	140	150	565
3. Control	205	195	810
4. Chronic myeloid leukaemia	745	440	17000
5. Chronic myeloid leukaemia	140	140	5400

TABLE II

RECOVERIES OF ADDED VITAMIN B_{12} FROM PACKED RED CELLS

Experiment No.	*Vitamin B_{12} added (µµg./ml. packed cells)*	
	250	500
	Vitamin-B_{12} activity recovered (µµg./ml. packed cells)	
1	185	425
2	240	480
3	170	400
4	295	375
5	395	415
6	310	455
	250	525
Mean	272	461

RESULTS

Recovery of Added Vitamin B_{12} from Packed Red Cells, and Reproducibility of Results

When vitamin B_{12} was added to seven samples of packed red cells, recoveries of between 68 and 158 per cent were obtained (Table II). The mean recovery with 250 µµg. of vitamin B_{12} added per ml. of packed cells was 109 per cent, and with 500 µµg. added per ml., 92 per cent.

A large sample of packed cells was divided into 12 portions of 2 ml. which were assayed singly at random intervals over a period of 6 months. The results varied between 175 and 395 µµg./ml. (mean 260, standard deviation 58).

Stability of the Vitamin-B_{12} Activity in Red Cells to Alkali

Extracts from five samples of packed red cells were made alkaline with N-NaOH solution, heated at 100° C. for 30 minutes and neutralized with N-HCl, and the residual vitamin-B_{12}

activity was assayed. This treatment destroyed 94 per cent or more of the vitamin-B_{12} activity (Table III).

Levels of Vitamin-B_{12} Activity in Packed Red Cells and Plasma

The results from the various groups of subjects are illustrated in Figs. 1 and 2 and summarized in Table IV.

Control subjects. The levels in red cells ranged from 110 to 500 µµg./ml. and in plasma from 150 to 1540 µµg./ml. All but three of the values from plasma were within the range of 150–1000 µµg./ml. found for serum from 123 normal subjects by Spray and Witts (1958), but

TABLE III

EFFECT OF TREATMENT WITH ALKALI ON THE VITAMIN-B_{12} ACTIVITY IN EXTRACTS OF PACKED RED CELLS

Sample No.	*Vitamin-B_{12} activity (µµg./ml. packed cells)*	
	Before alkali treatment	*After alkali treatment*
1	230	0
2	190	11
3	230	11
4	160	10
5	205	11

TABLE IV

VITAMIN-B_{12} ACTIVITY IN RED CELLS AND PLASMA IN NORMAL SUBJECTS AND VARIOUS DISEASE STATES

Diagnosis	*No. of patients*	*Vitamin-B_{12} activity (µµg./ml.)*			
		Packed red cells		*Plasma*	
		Observed range	*Mean*	*Observed range*	*Mean*
Controls	50	110–500	213	150–1540	603
Untreated pernicious anaemia	12	25–110	63	25–115	61
Latent pernicious anaemia	8	62–210	119	125–370	181
Hypochromic anaemia (treated)	5	90–140	127	100–275	220
Chronic myeloid leukaemia	11	110–440	228	1000–17,000	4470
Chronic lymphatic leukaemia	4	115–175	143	320–1120	560
Acute leukaemia	4	150–645	371	120–1480	598
Polycythaemia vera	10	75–200	136	270–1200	605

the mean was significantly higher than the mean of 450 µµg./ml. found previously for serum ($n = 171$, $t = 4.5$, $P < 0.001$). However, there was a highly significant correlation between the results from plasma and serum obtained from the same samples of blood on 20 subjects examined during the present study ($r = 0.98$, $P \ll 0.001$). The mean for these 20 samples of plasma was 219 µµg./ml. and for serum 206 µµg./ml. All the control subjects showed lower levels in the red cells than in the plasma.

Untreated pernicious anaemia. The concentration of vitamin B_{12} in the red cells varied from 25 to 110 µµg./ml., and that in the plasma varied from 25 to 115 µµg./ml. Five of the 12 results for red cells were higher than the corresponding levels in plasma.

Latent pernicious anaemia. These patients were studied because they showed some or all of the features of pernicious anaemia, including inability to absorb vitamin B_{12} normally, while they were in good health and no abnormalities could be found in their peripheral blood (Callender and Spray, 1962). Seven had shown low or equivocal levels of vitamin B_{12} in their serum for between 2 and 9 years before the present investigation. Four of the eight patients showed levels of vitamin B_{12} in the red cells below any found in the control group;

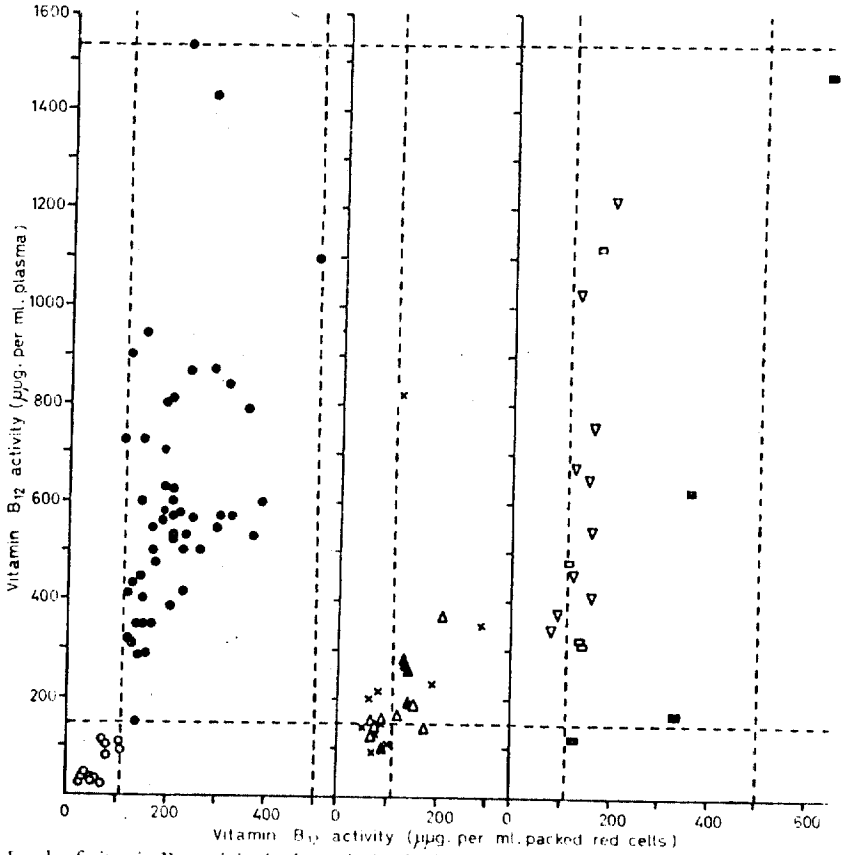

FIG. 1. Levels of vitamin-B_{12} activity in the packed red cells and plasma of different types of subject. ●, control subjects; ○, untreated pernicious anaemia; △, latent pernicious anaemia; ▲, hypochromic anaemia; ×, gastrectomy; □, chronic lymphatic leukaemia; ■, acute leukaemia; ▽, polycythaemia vera. The dotted lines indicate the upper and lower limits of the observed control ranges.

in the plasma only one result was above 200 µµg./ml. The mean value for plasma was significantly lower than the mean for the control subjects ($n = 56$, $t = 4.45$, $P < 0.001$), and the mean for the red cells was also significantly lower than the control mean ($t = 3.18$, $0.01 > P > 0.001$).

Hypochromic anaemia. These five patients had been treated with iron. They were studied because they showed some of the abnormalities associated with latent pernicious anaemia, such as achlorhydria or abnormal gastric biopsy appearances. They could not be classified as latent pernicious anaemia because of some such feature as a comparatively recent finding of normal absorption of vitamin B_{12}, but it is possible that their ability to absorb vitamin B_{12}

may have been deteriorating. Four showed values within the control range for vitamin B_{12} in both red cells and plasma, and the other showed low levels in both compartments.

Partial or total gastrectomy. Of the 10 patients studied, eight showed evidence of deficiency of vitamin B_{12} or folic acid as judged by the appearances of their bone marrow or peripheral blood, and two showed evidence of iron deficiency. The level of vitamin B_{12} in the red cells was below the observed lower limit of the control range in seven of these patients, but only four showed low values in the plasma.

Chronic myeloid leukaemia. These patients were studied because they are known to have high levels of vitamin B_{12} in the serum (Spray and Witts, 1958). Some were untreated and others had received treatment. All showed normal values for vitamin B_{12} in the red cells, while the plasma results ranged from 1000 to 17,000 µµg./ml.

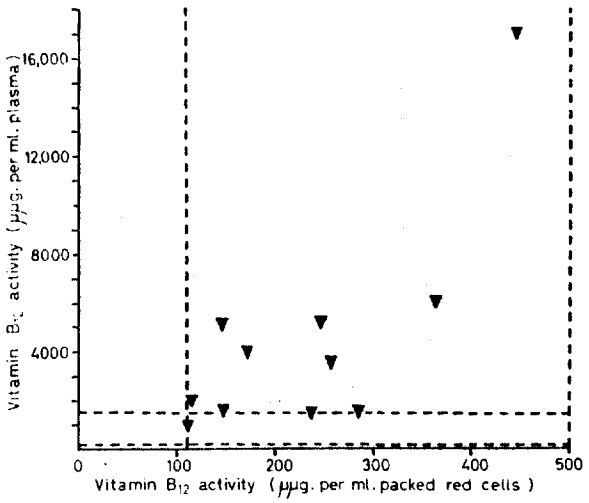

FIG. 2. Levels of vitamin-B_{12} activity in the packed red cells and plasma of patients with chronic myeloid leukaemia. The dotted lines indicate the upper and lower limits of the observed control ranges.

Chronic lymphatic leukaemia. Three of these patients were treated and one was untreated. All the values for vitamin B_{12} in both red cells and plasma were within the observed control ranges.

Acute leukaemia. Three patients who had received treatment all gave normal results for vitamin B_{12} in red cells; the values from plasma were 165, 625 and 120 µµg./ml. One patient who was studied before treatment had an abnormally high level in the red cells; the result from the plasma was near the upper limit of the control range.

Polycythaemia vera. All these patients were receiving treatment. Two of the results from red cells were below the control range, and the mean was significantly lower than the control mean ($n = 58$, $t = 2.92$, $0.01 > P > 0.001$). All the plasma values were within the observed control range.

DISCUSSION

Our data suggest that the method used for the assay of vitamin-B_{12} activity in packed red cells provides, within the limitations of microbiological assay procedures, a quantitative measure of the vitamin-B_{12} content of the cells. The almost complete destruction of the acti-

vity by treatment with alkali indicates that the activity is due to vitamin B_{12} and not to other substances which might stimulate the growth of the test organism.

Our results confirm the observation of Sobotka *et al.* (1960) that the concentration of vitamin B_{12} in the red cells of control subjects is lower than that in plasma. On average the level in the red cells of our control subjects was 35 per cent of that in the plasma; the corresponding figure in the patients with untreated pernicious anaemia was 103 per cent and in those with latent pernicious anaemia 66 per cent. This suggests that as deficiency of vitamin B_{12} develops, the level in the plasma falls faster than that in the red cells. If the level in red cells is regarded as a measure of that in other tissues, the relationship is comparable to that found in rats after total gastrectomy (Booth and Spray, 1960). The patients with hypochromic anaemia may be at an earlier stage of incipient vitamin-B_{12} deficiency than those with latent pernicious anaemia, so that the ratio might be expected to be higher than in the control subjects but less than in latent pernicious anaemia. The mean value of the five subjects studied was 58 per cent.

All the patients with chronic myeloid leukaemia had normal levels of vitamin B_{12} in the red cells while the concentration in plasma was often high. Mollin and Ross (1955) found normal amounts of vitamin B_{12} in the tissues of a patient who died with chronic myeloid leukaemia and who had a high level in the serum. The significantly lower mean level of vitamin B_{12} in the red cells of the patients with polycythaemia vera after treatment cannot be explained at present.

The patients who were studied after gastrectomy might have had deficiency of vitamin B_{12}, folic acid or iron, or any combination of these. The results have therefore not been grouped together for comparison with the control group. The expected findings of normal values in both red cells and plasma, or low values in both compartments, appeared in seven patients. The remaining two patients, together with two with polycythaemia vera, were anomalous in showing low values in red cells while the results from plasma were within the observed control range.

The significantly higher mean level of vitamin B_{12} in the plasma of our control subjects, as compared with the normal subjects studied by Spray and Witts (1958), is difficult to explain. Our control subjects were studied during 1961 and 1962, whereas the other group was studied in 1955–57. Minor changes have been made in the assay procedures during this period, particularly the use of different cultures of the test organism. This is because the organism sometimes fails to give satisfactory results after repeated subculture and a fresh culture must be used. Some improvement may have occurred in the general nutritional state of the population in the interval between the two sets of observations. The most recently reported normal range, determined by saturation analysis (Barakat and Ekins, 1963), was 330–1070 µµg./ml. (mean 611).

SUMMARY

A method is described for the estimation of vitamin-B_{12} activity in packed red cells by microbiological assay with *Lactobacillus leichmannii*. The accuracy of the method was assessed by recovery experiments and tests of the reproducibility of results.

The vitamin-B_{12} activity in the red cells of 50 control subjects ranged from 110 to 500 µµg./ml. The mean (213 µµg./ml.) was 35 per cent of the mean level in the plasma. In 12 patients with untreated pernicious anaemia the levels in red cells ranged from 25 to 110 µµg./ml., and the mean (63 µµg./ml.) was 103 per cent of the mean level in the plasma. Eight

patients with 'latent pernicious anaemia' gave values in red cells from 62 to 210 µµg./ml. (mean 119 µµg./ml., or 66 per cent of the mean level in the plasma), while the levels in the red cells of five patients with hypochromic anaemia and some but not all of the features of latent pernicious anaemia were between 90 and 140 µµg./ml. (mean 127 µµg./ml.).

Seven out of 10 patients who had had a gastrectomy showed subnormal levels of vitamin-B_{12} activity in red cells. In chronic myeloid leukaemia all the results in red cells were within the control range in spite of high values in the plasma, and in chronic lymphatic leukaemia and acute leukaemia the levels in red cells were usually normal. The mean value for the red cells of treated patients with polycythaemia vera was significantly lower than the mean for control subjects.

ACKNOWLEDGMENTS

We are grateful to the technicians in the haematology laboratory of this Department for help in the collection of specimens and for providing haematological data on the patients included in this study; and to Professor L. J. Witts, in whose department this work was done.

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Lancet 1969, 50

VITAMIN B_{12} AND FERTILITY

SIR,—The preliminary communication of Watson [1] and the letters that followed [2] prompted us to make an additional observation. All the evidence indicates that the impaired fertility that accompanies anæmia may be corrected with vitamin B_{12}, as has been shown by Dr. Jackson and his colleagues (Dec. 2, p. 1159) and in the letter that followed by Dr. Varadi (Dec. 16, p. 1305). Various mechanisms for the role of this vitamin in this connection have been suggested: Tomaszewski et al.[3] have noted its relation to androgens; Adams [4] showed that low semen-vitamin-B_{12} levels preceded anæmia by several months, and suggested that the vitamin deficiency may be responsible for infertility. These workers have suggested that male infertility might be successfully treated with this vitamin.

We have investigated two men with poor seminal quality (possibly due to heavy smoking and drinking [5]) which improved greatly during a seven-month course of weekly injections of 30 µg. vitamin B_{12}. In both cases all hæmatological (red and white blood-cells, Hb, and hæmatocrit) and thyroid findings were normal. Semen samples were obtained at weekly intervals, and there was no change in either alcohol or cigarette consumption during the period of investigation. There was a baseline period of six weeks, followed by a drug period of six months, so that a total of about 30 semen samples were obtained from each man.

Case 1.—A 35-year-old White man with three children. Alkaline-phosphatase and bilirubin levels were in the high normal range, and his serum-glutamic-pyruvic-transaminase was somewhat raised (64·0 units). His sperm count was 3 million per ml. (range 1–16 million) with complete necrospermia in his baseline samples and 9% normal oval forms, and about half of the cells were immature. (We wish to thank Dr. John MacLeod for evaluating the morphological characteristics in these specimens.) After six weeks' treatment his count and motility rose steadily to a final average of 140 million per ml. and 80% motility, for the last month. The immature forms completely disappeared and there are now over 50% normal oval sperm cells. All liver-function tests are now in the normal range.

Case 2.—A 50-year-old White man with two children. All liver-function tests were in the normal range. During the baseline period his sperm count was 32 million per ml. (range 20–43 million) with 3 ml. seminal volume. Motility was 10% with 24% normal forms and 25% immature forms. After seven weeks' treatment his count increased steadily to a level of 150 million with 75% motility and 60% normal forms as averages for the final month. Immature cells dropped to 5%.

These two cases confirm the work of others on the influence of vitamin B_{12} on fertility; but since most of the clinical reports are concerned with impaired infertility accompanying anæmia, they suggest that vitamin B_{12} might be beneficial in

1. Watson, A. A. *Lancet*, 1962, ii, 644.
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the treatment of low fertility in other cases. The possibility of liver damage is supported by the abnormal tests in case 1. It is difficult, however, to postulate a role for vitamin B_{12} in liver damage; and since this vitamin has many biochemical functions [6] (including possibly the reduction of ribonucleotide to deoxyribonucleotide) we should not speculate on its possible role in the production, maturation, and motility of spermatozoa. These processes could possibly be influenced by cobalt, which can stimulate erythropoiesis.[7]

Further investigations on the effect of vitamin B_{12} on normal subjects with poor seminal quality, and heavy drinkers or alcoholics, seem indicated.

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Identification of a New Vitamin B_{12} Binder (Transcobalamin III) in Normal Human Serum

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Summary. Fractionation of vitamin B_{12} binders in a normal human serum pool showed a distribution of 92% Transcobalamin II (TCII) and 8% Transcobalamin I (TCI) on DEAE-cellulose chromatography. Separation of the same serum pool using gel filtration gave 74% TCII and 26% TCI. This 18% discrepancy could be due either to massive contamination of the DEAE-cellulose TCII peak with TCI, or to the presence of a high molecular weight binder which elutes with TCII on DEAE-cellulose, and with TCI on gel filtration.

Refractionation of the TCII peak showed it to be relatively uncontaminated with TCI. Removal of the low molecular weight binders with charcoal consistently gave a peak in the TCII area on DEAE, which subsequent gel filtration analysis showed to be of high molecular weight. This new binder is further distinguished from TCI by the observation that its vitamin B_{12} binding capacity does not increase to the same extent as TCI on fasting.

Since this binder could be demonstrated in 50 different normal sera, it is clearly not a pathological binder, and should be called Transcobalamin III (TCIII).

Fractionation of vitamin B_{12} enriched serum by DEAE and CM-cellulose chromatography has suggested that there are two main vitamin B_{12} binders present in normal human serum. Electrophoretic separation has demonstrated these to be an α_1-globulin, and a β-globulin (Fahey *et al*, 1958; Hall & Finkler, 1965). These were designated by Hall & Finkler (1965) as Transcobalamin I (TCI) and Transcobalamin II (TCII) respectively. Gel filtration of serum has also resolved the vitamin B_{12} binding proteins into two fractions, which have been designated TCI and TCII, the former having a higher molecular weight than the latter (Hom *et al*, 1966; Hom & Oleson, 1967; Hom & Ahluwalia, 1968). It has been our consistent observation that fractionation of the vitamin B_{12} binders in serum into TCI and TCII is different on DEAE-cellulose and gel filtration. We have shown that this discrepancy is due to a hitherto unrecognized serum binder which fractionates with the so called TCII peak on DEAE-cellulose but being of large molecular weight, fractionates with the TCI peak on gel filtration. Since this new binder can now be demonstrated always to be present in normal human serum we have designated it Transcobalamin III (TCIII).

Combination of both these methods has resolved the three binders, and has shown that the new binder complexes vitamin B_{12} in amounts of up to 20% of the total unsaturated vitamin B_{12} binding capacity (UBBC). It has also been shown that the amount of unsaturation of this

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binder varies with the vitamin B_{12} status of the serum, which suggests that this binder plays an important role in the transport of vitamin B_{12}. A screening method has been proposed, based on the methods of previous workers (Retief *et al*, 1967; Lawrence, 1969), which allows rapid assay of the new binder in normal and pathological sera.

MATERIALS AND METHODS

Subjects. Normal serum was obtained from 10 men and 9 women ranging in age from 22 to 24. None had any haematological or gastroenterological problems as far as was known, and all had vitamin B_{12} and folic acid levels in the normal range. Sera were collected (a) after a 12 hr fast, and (b) 4 hr after a normal lunch which had been preceded 4 hr earlier by a normal breakfast. All sera were stored at −20°C until they were used.

Vitamin B_{12}. A working solution in isotonic saline of ^{57}Co-labelled cyanocobalamin (^{57}Co-B_{12}) was prepared by mixing ^{57}Co-B_{12} (100 μCi/μg, Radiochemical Centre, Amersham) containing 10 ng/ml, with ^{57}Co-B_{12} containing 30 ng/ml (1 μCi/μg), in a one to one ratio. This gave a ^{57}Co-B_{12} working solution of 20 ng/ml in isotonic saline.

DEAE-cellulose chromatography was carried out as described by Retief *et al* (1967), with the following modifications: serum was saturated with ^{57}Co-B_{12} by addition of 0.1 ml (2000 pg) of the ^{57}Co-B_{12} working solution per ml of serum. Free ^{57}Co-B_{12} was removed from the serum using albumin-coated charcoal, prepared according to Gottlieb *et al* (1965). The charcoal suspension (1 ml per ml of serum) was filtered by membrane filtration and the powder added to the serum following incubation of the serum with ^{57}Co-B_{12}. The free ^{57}Co-B_{12} was rapidly adsorbed onto the charcoal after mixing, and the serum was refiltered by membrane filtration (Bloomfield & Scott, 1971). Dialysis was carried out against 0.02 M phosphate buffer, pH 6.3, for 12 hr at 4°C. Chromatography was then carried out using DE23 anion exchange cellulose (Whatman, Balston Ltd, England) by application of 1.1 ml serum to the column. One ml samples were collected at a flow rate of 12 ml/hr, the eluant being changed from 0.06 M phosphate buffer to 1.0 M NaCl after tube 14.

Gel filtration was performed through 2.5 × 45 cm columns of Sephadex G-200 (Pharmacia, Uppsala, Sweden), collecting 4 ml samples at a flow rate of 15 ml/hr. Sephadex G-200 was equilibrated in 0.04 M phosphate buffer, pH 7.4, containing 0.5 M NaCl. The serum was labelled with ^{57}Co-B_{12} as outlined above. The void volume averaged 60 ml and three protein peaks, representing macroglobulin, γ-globulin and albumin, were recorded respectively in samples eluted thereafter. The high molecular weight vitamin B_{12} binding proteins appeared between the γ-globulins and albumin, and the low molecular weight vitamin B_{12} binders appeared after the albumin peak. The eluates off Sephadex G-200 and DEAE were investigated as follows:

1. Radioactivity was determined in an automatic gamma counter (Nuclear Chicago, Model 4216), and converted to pg ^{57}Co-B_{12} per eluate, by comparison to a radioactive standard.

2. Optical density (O.D.) at 280 mμ was determined in a Unicam SP500 spectrophotometer. Samples off DEAE were diluted 1 : 10 in a few cases to give an indication of total protein.

For removal of the low molecular weight vitamin B_{12} binders, the method outlined by Lawrence (1969) was employed. For convenience, the percentages given below represent the percentage of ^{57}Co-B_{12} bound to the different Transcobalamins, the total UBBC being 100% of the radioactivity.

RESULTS

A serum pool, prepared from the 19 fasted sera, was labelled with ^{57}Co-B_{12} as described above.

As expected, fractionation of DEAE (Fig 1) consistently resulted in two peaks, the first representing 92% of the radioactivity (TCII), and the second representing 8% of the radioactivity (TCI). However, while fractionation of this same serum pool on Sephadex G-200 also gave two peaks, the high molecular weight peak (TCI) contained 26% and the low molecular weight peak (TCII) contained 74% of the radioactivity (Fig 2).

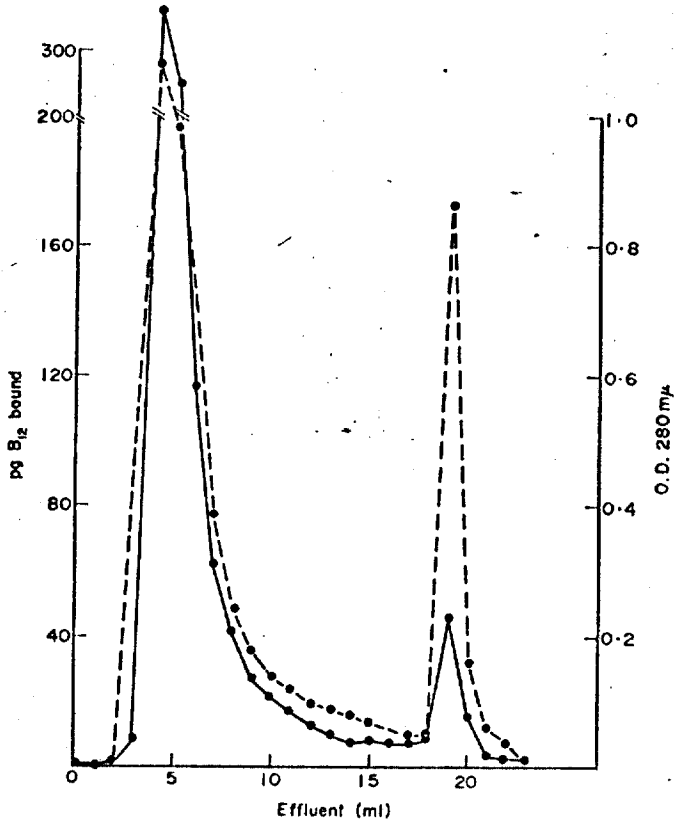

FIG 1. DEAE-cellulose fractionation of 1.1 ml ^{57}Co-B_{12} saturated serum; continuous line, ^{57}Co-B_{12}; broken line, O.D.

There are two possible explanations for this 18% discrepancy between the two methods of fractionation: (1) that TCII as fractionated on DEAE is contaminated to the extent of 18% with high molecular weight TCI or (2) that another binder exists, which fractionates with TCII on DEAE, but unlike TCII is of high molecular weight.

The first DEAE peak (TCII) was refractionated under identical conditions, following dialysis overnight against 0.02 M phosphate buffer, pH 6.3, and reduction of volume by ultrafiltration under pressure (Fig 3). It was found that while TCII was contaminated with TCI, this contamination never represented more than 2% (Fig 3). It thus seems highly unlikely that the original TCII peak could have been contaminated with 18% TCI. When the TCII peak, which had now been fractionated twice on DEAE, was refractionated on G-200, it contained not 18%, but 17.5% high molecular weight binder (Fig 4). This latter 17.5% apparently consisted of a new binder, uncontaminated with TCI, and separated from TCII. Since this binder occurs in normal human serum, and is different from the two known binders TCI and TCII, we have called it TCIII.

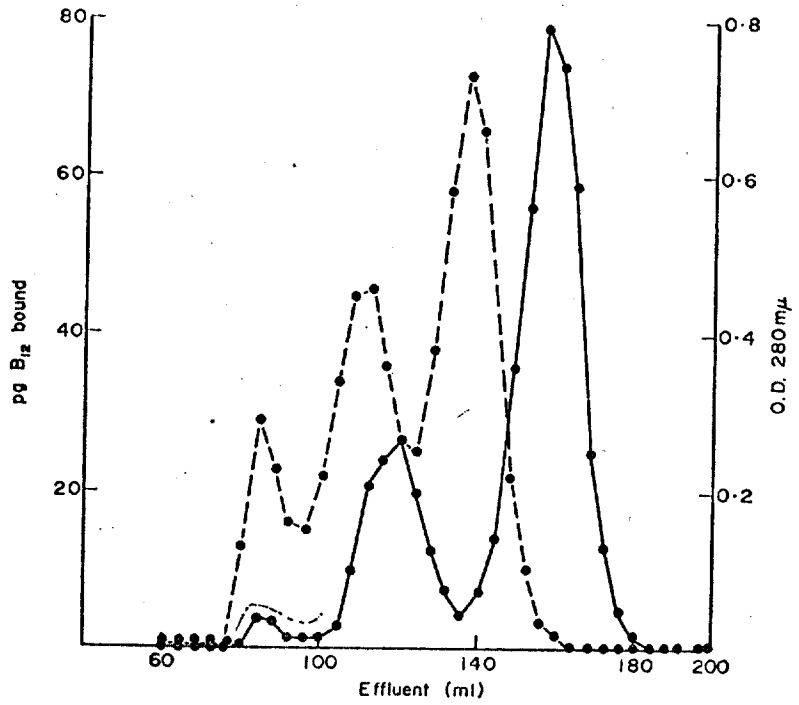

FIG 2. Sephadex G-200 fractionation of 0.55 ml ^{57}Co-B_{12} saturated serum; continuous line, ^{57}Co-B_{12}; broken line, O.D.

The above experiments were repeated several times both on the single fasted pool and on other individual sera. However, because of the length of the procedure, it was decided to investigate the possibility of simplifying it for routine screening. It has previously been reported that all of the low molecular weight vitamin B_{12} binding protein of serum is removed by treatment with uncoated charcoal (Lawrence, 1969).

The serum pool was treated with uncoated charcoal and labelled with ^{57}Co-B_{12} as before. The distribution of the radioactivity was examined on Sephadex G-200 (Fig 5). Only the low molecular binders were removed by this procedure leaving 24% of the radioactivity representing the high molecular weight binders still in solution. The same serum when run on DEAE showed a distribution of 6% appearing in the position of TCI, and 18% in the TCII

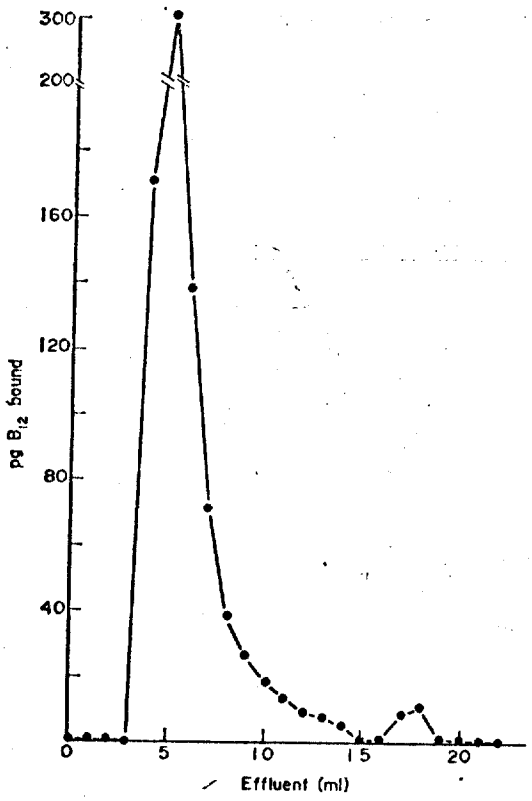

FIG 3. DEAE-cellulose re-run of TCII peak from 1.1 ml of ^{57}Co-B_{12} saturated serum previously fractionated on DEAE-cellulose.

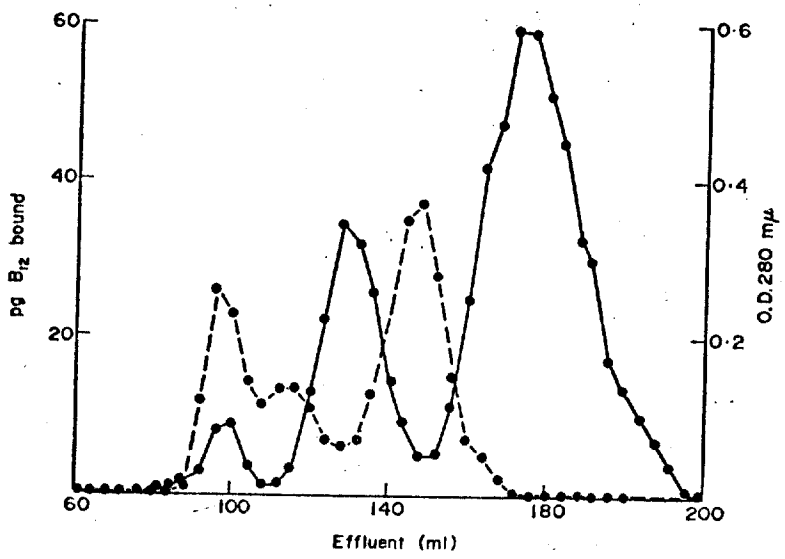

FIG 4. Sephadex G-200 fractionation of TCII peak after second fractionation on DEAE-cellulose; continuous line, ^{57}Co-B_{12}; broken line, O.D.

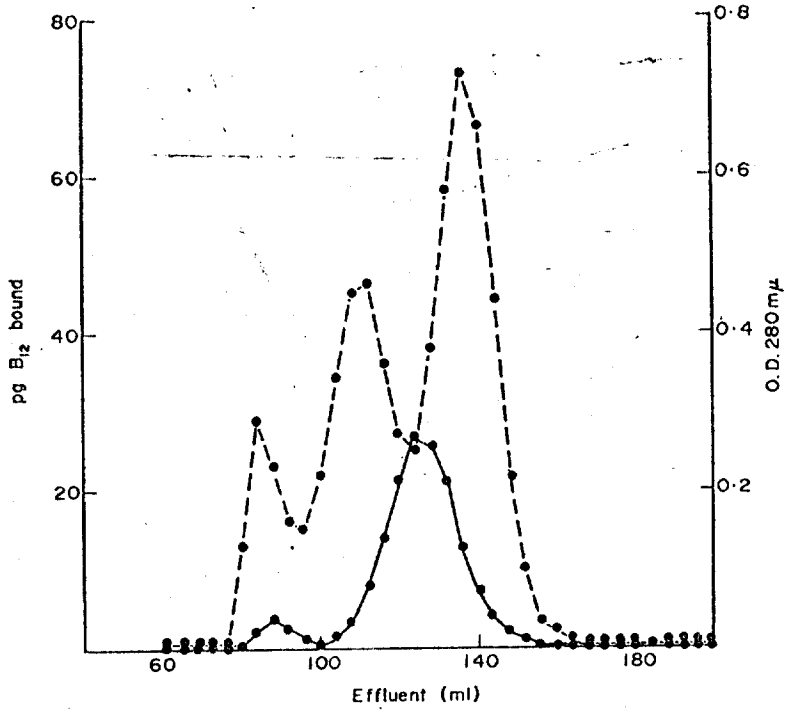

FIG 5. Sephadex G-200 fractionation of 0.55 ml ^{57}Co-B_{12} saturated serum after treatment with uncoated charcoal; continuous line, ^{57}Co-B_{12}; broken line, O.D.

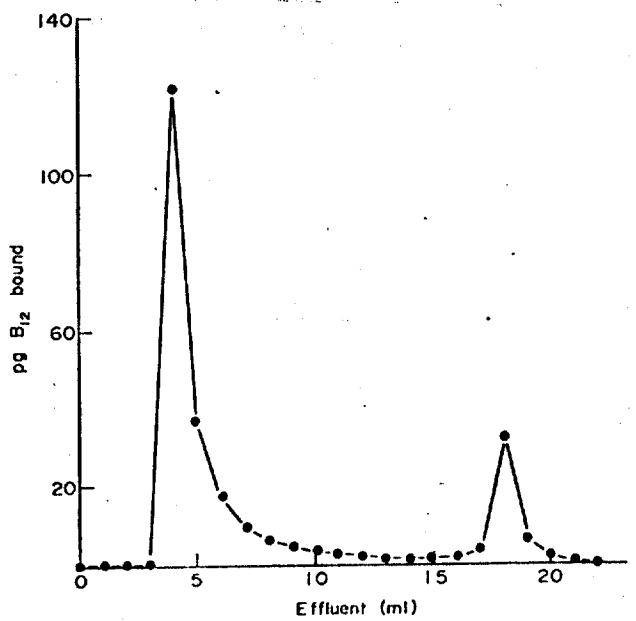

FIG 6. DEAE-cellulose fractionation of 1.1 ml ^{57}Co-B_{12} saturated serum which had previously been treated with uncoated charcoal.

position (Fig 6). However, TCII should have been removed, and the 18% peak was found upon analysis on Sephadex G-200 to be devoid of low molecular weight material (Fig 7).

Our interpretation of these results is that, after charcoal treatment to remove the low molecular weight binders, the first peak appearing on DEAE fractionation (in the position of TCII) is the new TCIII binder. It may be contaminated with a small amount of TCI, but this always represents a tiny proportion of the total. Using treatment with uncoated charcoal the low molecular weight binders were removed from the 19 fasted and 19 fed sera. They were then labelled with ^{57}Co-B_{12} as outlined before. All samples were run on DEAE-cellulose, as

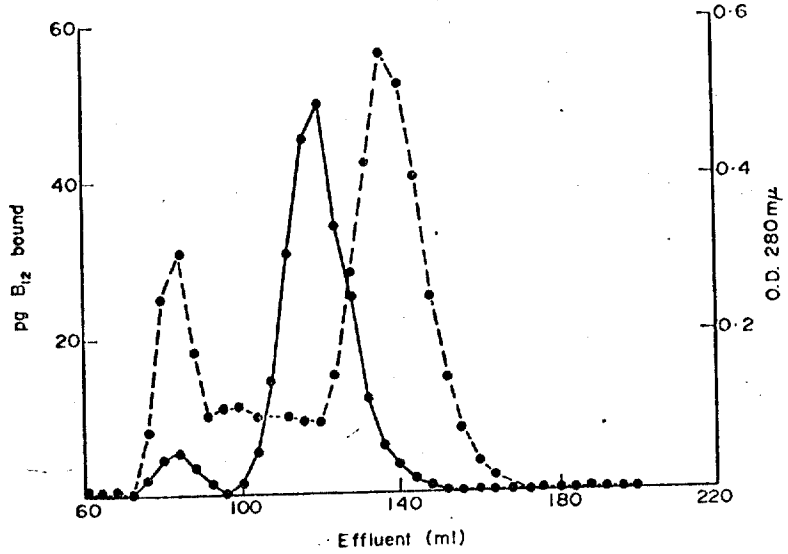

FIG 7. Sephadex G-200 fractionation of the peak which had eluted as TCII on DEAE cellulose; continuous line, ^{57}Co-B_{12}; broken line, O.D.

described in the Materials and Methods section, except that total peaks were collected by eluting three 5 ml fractions using the buffer eluant, followed by two 5 ml fractions using 1 M NaCl. The radioactivity was measured and expressed as pg ^{57}Co-B_{12} per total peak in each case (Table 1). The first peak contained ^{57}Co-B_{12}/TCIII complex and the second peak contained ^{57}Co-B_{12}/TCI complex. The total UBBC was determined for each serum by the method of Gottlief *et al* (1965) using ultrafiltration instead of centrifugation (Bloomfield & Scott, 1971). The results demonstrated that the total UBBC was increased by fasting. They also showed that while the UBBC of TCIII was increased by fasting, the percentage increase in UBBC of TCI was far greater in the fasted state. These results confirm that the high molecular weight B_{12} binder in the first peak is not TCI contamination, for if it were, the two peaks would show the same relative increase on fasting.

DISCUSSION

The fact that the two methods for fractionation of the vitamin B_{12} binders consistently yielded different results led to the belief that either the TCII peak was contaminated with

TABLE I. Distribution of human UBBC between the different Transcobalamins in (a) normal and (b) fasted sera on DEAE-cellulose fractionation after treatment with uncoated charcoal

Subject		Total UBBC (pg/ml)	TCIII		TCI		TCIII and TCI		TCII*	
			pg/ml	%	pg/ml	%	pg/ml	%	pg/ml	%
P.E.	a	922.3	204.5	22.1	64.3	6.9	268.3	29.0	654.0	71.0
	b	1328.9	221.9	16.7	238.0	17.9	459.9	34.6	869.0	65.4
J.S.	a	994.7	122.1	12.3	71.2	7.1	193.3	19.4	801.4	80.6
	b	1527.1	166.3	10.9	198.0	13.0	364.3	23.9	1162.8	76.1
A.C.	a	1090.8	114.7	10.5	70.4	6.5	185.1	17.0	905.7	83.0
	b	1674.0	346.6	20.7	276.3	16.5	622.9	37.2	1051.1	62.8
L.H.	a	1206.9	131.3	10.9	55.3	4.6	186.6	15.5	1020.3	84.5
	b	1197.7	230.0	19.2	104.7	8.7	334.7	27.9	863.0	72.1
T.B.	a	1002.2	177.3	17.7	75.1	7.5	252.4	25.2	749.8	74.8
	b	1749.6	196.6	11.2	178.4	10.2	375.0	21.4	1374.6	78.6
N.F.	a	959.6	199.3	20.8	88.9	9.3	288.2	30.1	671.4	69.9
	b	1871.1	210.2	11.2	496.1	26.5	706.3	37.1	1164.8	62.3
C.B.	a	984.9	134.9	13.7	95.7	9.7	230.6	23.4	754.3	76.6
	b	1708.6	232.4	13.6	411.6	24.1	644.0	37.7	1064.6	62.3
B.P.	a	1132.0	249.4	22.0	35.6	3.0	285.0	25.0	847.0	75.0
	b	2107.1	438.8	20.8	269.9	12.8	708.7	33.6	1398.4	66.4
A.J.	a	1309.5	133.3	10.2	54.3	4.1	187.6	14.3	1121.9	85.7
	b	1423.4	183.8	12.9	280.5	19.7	464.3	32.6	959.1	67.4
C.L.	a	1064.9	116.7	11.0	171.9	16.1	288.6	27.1	776.3	72.9
	b	1989.9	176.0	8.8	290.5	14.6	466.5	23.4	1523.4	76.6
B.G.	a	938.5	182.5	19.4	60.3	6.4	242.8	25.8	695.7	74.2
	b	1327.3	182.9	13.8	257.0	19.4	439.9	33.2	887.4	66.8
T.McC.	a	897.1	161.2	18.0	31.3	3.5	192.6	21.5	705.1	78.5
	b	1187.5	184.7	15.6	225.1	18.9	409.8	34.5	777.7	65.5
A.O'C.	a	1332.2	127.8	9.6	54.6	4.1	182.4	13.7	1149.8	86.3
	b	2124.9	199.2	9.4	211.7	9.9	410.9	19.3	1714.0	80.7
E.O'C.	a	1320.6	143.7	10.9	63.2	4.8	206.9	15.7	1113.7	84.3
	b	1431.0	242.0	16.9	247.3	17.3	489.3	34.2	941.7	65.8
M.D.	a	1343.3	265.0	19.7	74.6	5.6	339.6	25.3	1003.7	74.7
	b	1532.2	162.8	10.6	386.6	25.2	549.4	35.8	982·8	64.2
G.W.	a	1037.3	133.2	12.8	69.4	6.7	202.6	19.5	834.7	80.5
	b	1079.5	177.2	16.4	308.2	28.6	485.4	45.0	594.1	55.0
H.M.	a	961.2	180.0	18.7	72.2	7.5	252.2	26.2	709.0	73.8
	b	1258.2	213.9	17.0	225.2	17.9	439.1	34.9	819.1	65.1
M.K.	a	1242.4	193.9	15.6	80.6	6.5	274.5	22.1	967.9	77.9
	b	1416.4	247.4	17.0	196.4	14.0	443.8	31.0	972.6	69.0
D.G.	a	939.6	130.8	13.9	27.8	3.0	158.6	16.9	781.0	83.1
	b	1239.3	139.9	11.3	298.3	24.1	438.2	35.4	801.1	64.6
Mean values	a	1088.4	163.2	15.3	69.3	6.4	232.5	21.7	855.9	78.3
	b	1535.5	218.6	14.4	268.4	17.9	487.0	32.3	1048.5	67.7

* By subtraction of TCI and TCIII from total UBBC.

TCI or that there was a third binder present which was eluted with TCII on DEAE-cellulose fractionation, and with TCI by gel filtration. When the TCII peak was re-run on a fresh column, less than 2% of the radioactivity was left to be eluted by 1 M NaCl (Fig 3). It would be expected that a second run should have separated off the TCI contaminant due to the smaller amount being present. Gel filtration showed that the TCII peak, which had been run twice through a DEAE-cellulose column, still contained about 17.5% of a high molecular weight component (Fig 4). We believe this to be a semi-purified binder not previously described. Since this binder was found in the 50 sera investigated, it is obviously a normal binder and should be called Transcobalamin III.

Treatment of the pooled serum with uncoated charcoal showed a consistent removal of 74–76% of the radioactivity leaving about 24% ^{57}Co-B_{12} which was shown to be bound to high molecular weight binder only (Fig 5). The fact that 17.5% of this was eluted with 0.06 M phosphate buffer indicated that this was not TCI because elution of this binder does not take place at this buffer concentration (Fahey *et al*, 1958).

Fasting causes an increase in the UBBC of the serum binders (Table I). The mean distribution of the three serum binders (expressed in pg/ml) relative to the total UBBC was; UBBC, 1088.4 (100%); TCIII, 163.2 (15.3%); TCII, 855.9 (78.3%); TCI, 69.3 (6.4%). Upon fasting these values change to; UBBC, 1535.5 (100%); TCIII, 218.6 (14.4%); TCII, 1048.5 (67.7%); TCI, 268.4 (17.9%). It is also apparent from Table I that the alterations effected in levels of TCI and TCII by fasting differ markedly from TCIII.

Whether the TCIII described in this paper is similar to the foetal binder described by Kumento and his co-workers (Kumento *et al*, 1967) or to that found in polycythaemia vera (Hall & Finkler, 1969) is currently under investigation.

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A PHYSICIST'S INTERPRETATION OF SOME ASPECTS OF VITAMIN B_{12} METABOLISM AND ITS USE TO ROUTINELY ESTIMATE TOTAL-BODY B_{12}

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It is with great deference that a physicist offers an interpretation of material derived from clinical studies In mitigation, a serious attempt has been made to understand some of the clinical problems of our medical colleagues to improve the effectiveness of collaboration. As would be expected, a physicist lacks a detailed knowledge of vitamin B_{12} metabolism and of the pertinent literature, but this detachment may avoid preconceptions. Although the interpretation will consequently be oversimplified, it might be adequate for practical purposes, particularly for other nonspecialists.

Many studies of vitamin B_{12} metabolism are based on the simple hypothesis of effective "equilibrium" in the body between administered doses of tracer B_{12} and native B_{12}, which are then assumed to behave identically. When this assumption was disputed (*1–4*), it was necessary to consider its basis more closely and to demonstrate whether it was valid or not. Despite an extensive literature, no comprehensive examination of available data appears to have been undertaken in this particular respect. A preliminary report (*5*) concluded that there was clinical evidence in favor of the simple hypothesis of "equilibrium" and that it was an adequate approximation for most clinical purposes. A more detailed consideration of this hypothesis is presented here from which an interpretation of some aspects of vitamin B_{12} metabolism has been developed.

METABOLIC "EQUILIBRIUM" OF VITAMIN B_{12} AND ITS IMPLICATIONS

If metabolic "equilibrium" is established between tracer and native B_{12}, then its implications can be related to the available experimental data. This infers the following five points:

1. Vitamin B_{12} in the body is effectively in a single compartment. A kinetic analysis by Reizenstein and colleagues (*3,4*) chose initially three compartments but, paradoxically, showed that 99.3% of B_{12} was in a single compartment while a further 0.3% had been allocated to a second compartment before the analysis was carried out. It was not demonstrated whether 99.3 or 99.6% was significantly different from 100%, presumably because an analog computer was used. For most practical purposes, it seems reasonable to assume that the difference is not significant and that to a first approximation, all of the body B_{12} is effectively in a single compartment.

2. The specific activity of significant tissues should be the same. When "equilibrium" is established after administration of B_{12} labeled with a radionuclide, the specific activity (mμCi/gm vitamin) should be the same in all relevant tissues. A significant correlation ($r = 0.84$) was obtained between the concentration of labeled and native B_{12} in the tissues of a rooster, pig, and calf which were slaughtered only 5, 7, and 16 days, respectively, after the final injection of tracer (*6*). Similar findings have been reported in dogs (*7*) and in rats (*8,9*).

3. The excretion rate should be adequately described by a single exponential term. Using whole-body monitors, it has been possible to measure the body turnover of labeled vitamin B_{12} over periods of up to 2 years or more. The excretion rate could be adequately described by a single exponential term (*10–14*), and in our studies (*15,16*) there was a standard deviation of about 10%. We further showed (*16*) that the excretion rate did not change significantly after about 1 week postadministration of a tracer dose (0.1 μg i.v.) of B_{12}.

4. Excretion rates should correspond with B_{12} turnover rates in other tissues. Whole-body excretion rates (0.1–0.2%/day) agree well with hepatic clearance rates (0.08–0.46%/day) (*17–19*) and with long-term plasma turnover rates (0.1%/day) (*20*). In a recent study (*21*), long-term whole-body excretion rates were compared with plasma turnover

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in the same patients following massive intravenous doses of vitamin B_{12}. Good agreement was obtained.

The rate of loss of nonlabeled B_{12} in feces was estimated indirectly by microbiological assay of bile (*22*). A rate of only 0.03%/day was obtained. However, this value is probably an underestimate of loss from the body since it ignores both urinary losses and the possibility that much of the vitamin may have been in a microbiologically inactive form (*20*).

5. The amount of B_{12} lost daily from the body as estimated by the simple model should correspond with the daily intake. At "equilibrium," the daily loss of B_{12} (μg) is obtained simply by multiplying the excretion rate (0.1–2%/day) by the total-body content of B_{12} (1,000–3,500 μg). The daily loss is then about 1–7 μg/day which is in agreement with an estimated dietary absorption of 2–9 μg B_{12}/day (*12*).

The simple assumption of "equilibrium" between tracer and native B_{12} and its implications seem to be in reasonable accordance with available clinical and experimental evidence. On the other hand, the final exponential term by kinetic analysis (0.036%/day) (*3,4*) agrees only with the estimated loss of nonlabeled B_{12} (*22*) which is itself evidently an underestimate for the reasons discussed above.

EXTENSION OF THE HYPOTHESIS OF "EQUILIBRIUM"

If the hypothesis of "equilibrium" is accepted, then an interpretation, which unifies and is supported by previously isolated findings from several disciplines, can be developed for other aspects of vitamin B_{12} metabolism as is shown diagrammatically in Fig. 1. The hypothesis is that vitamin B_{12} is taken into the body primarily as hydroxocobalamin, with possibly some unconverted or unhydrolyzed coenzyme B_{12} and cyanocobalamin and converted to, stored, utilized, and transferred to the excretory system in a common form, possibly as coenzyme B_{12}. This simple concept obviously requires justification and supporting evidence although the specialist may consider that certain aspects have been well established already in his particular field of expertise.

There are several analog forms of vitamin B_{12}, the most important being hydroxocabalamin, cyanocobalamin, methylcobalamin, and coenzyme B_{12} (α-(5:6-diamethylbenziminazolyl) 5′-deoxyadenosyl cobamide). The bulk of vitamin B_{12} in the body is evidently in the coenzyme form (*23,24*) and will be primarily in this form in raw meats from animals, which constitute the major dietary source of the vitamin. However, coenzyme B_{12} is readily hydrolyzed to hydroxocobalamin and will generally be in the latter form in food

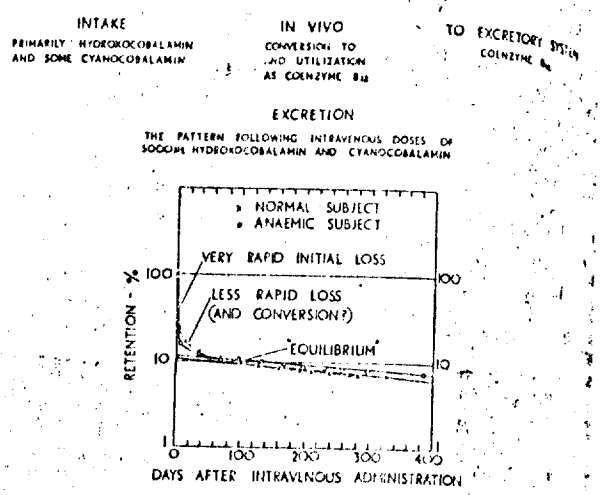

FIG. 1. Schematic representation of vitamin B_{12} metabolism. Analog forms of vitamin at various metabolic stages are indicated according to present interpretation. Observed excretion pattern following large intravenous doses of vitamin is shown with proposed explanation of metabolic processes involved.

since few meats are eaten uncooked. The conversion or reconversion in vivo of hydroxocobalamin to coenzyme B_{12} has been demonstrated (*25,26*). As indicated above, there is evidence that the vitamin is stored and used in the body as coenzyme B_{12}. It would then follow that whatever form of the vitamin was administered, following "equilibrium" the excretion rate should be the same since only a common form, possibly coenzyme B_{12}, reaches the excretory system and is subsequently lost to the body. It would not necessarily follow that the vitamin B_{12} will be in this form in excreta since there is evidence (*27*) that conversion can take place in urine after excretion by the kidney and an analagous situation may pertain in losses, via bile, in feces. In this case, the microbiological activity of coenzyme B_{12} in vitro would not contradict the present hypothesis and the suggestion (*20,22*) that only a fraction of excreted vitamin B_{12} is microbiologically active.

On the basis of the simple "equilibrium" model some quantitative confirmation of the present hypothesis can be derived:

1. The daily absorption (μg) should equal the conversion rate of hydroxocobalamin: coenzyme B_{12} and the daily loss. As discussed earlier, the daily loss estimated from the model agreed with the daily absorption, amounting to 1–10 μg/day.

Unfortunately, few data are available describing the amount of hydroxocobalamin or cyanocobalamin converted daily to coenzyme B_{12} (*25,26*). Uehleke and colleagues (*26*) studied the in vivo conversion in rats of the analogs to coenzyme B_{12} up to 24 hr after parenteral administration of 0.1 μg of B_{12} dose. The ranges obtained were 6.3–11.4 mμg hydroxo

obalamin per day and 2.1–3.0 mμg cyanocobalamin er day. To permit a crude extrapolation to man and to facilitate comparison with the findings of Pawelkiewicz and colleagues (*25*), these ranges can be expressed from the data given as 32–76 mμg/kg body weight/day and 11–20 mμg/kg body weight/day, respectively. The equivalent conversion rate of hydroxocobalamin in man would be about 2–5 μg/day which is similar to the daily absorption and excretion.

In rabbits (*25*) the in vivo conversion of cyanocobalamin amounted to 600 mμg/kg body weight/day, and a similar value could be deduced from parallel in vitro studies (*25*) using slices of human liver and kidneys. This value is higher than that found by Ochino et al (*26*), possibly because a comparatively large dose of 10 μg cyanocobalamin/kg body weight was injected into the rabbits and in vitro 6 μg per 5–10 gm liver or kidney was used. Although this explanation is speculative, if it were correct, it might imply that the rate of conversion can be increased in response to a large dose of B_{12} in excess of the normal daily intake.

Despite the obvious uncertainties involved in the extrapolation of animal data to man, it is encouraging that the values obtained tend to support the hypothesis and that the lower estimates of the conversion rate would be compatible with the estimated daily balance of vitamin B_{12} in man.

2. The metabolic pattern of large intramuscular doses of hydroxocobalamin and cyanocobalamin should be qualitatively similar, but the quantity retained will be roughly proportional to the conversion rates of these analogs to coenzyme B_{12}. The metabolic pattern of a similar dose of coenzyme B_{12} will differ from that of the other analogs. The retention of 1,000 μg intramuscular doses of hydroxocobalamin, cyanocobalamin, and coenzyme B_{12} has been studied by whole-body monitoring (*28*). The mean percentage retentions at 3 and 28 days are summarized in Table 1. The ratio of hydroxocobalamin: cyanocobalamin retained at 3 days is about 3:1 and is a similar value at 28 days. Their rates of conversion (μg/day) to coenzyme B_{12} are also in the ratio of about 3:1 (*26*). This suggests that the metabolism of these analogs is similar. The difference in the absolute amount retained may be explained in simple terms if the conversion rates are proportional to the respective number of reaction sites available for conversion to be effected; viz. preferential conversion of hydroxocobalamin will be associated with proportionately greater retention.

The ratio of coenzyme B_{12}:cyanocobalamin retained at 3 days is about 2.7:1, but at 28 days the ratio is significantly less, 1.4:1. Evidently the metabolism of coenzyme B_{12} is different from that of the other analogs until "equilibrium" is reached. This would be expected if hydroxocobalamin and cyanocobalamin must undergo conversion, while coenzyme B_{12} is already in the form in which the vitamin is used, stored, and passed to the excretory system.

TABLE 1. METABOLISM OF 1,000 μg INTRAMUSCULAR DOSES OF VITAMIN B_{12} ANALOGS

Analog	Mean retention %	
	At 3 days	At 28 days
Hydroxocobalamin	42.1	32.5
Cyanocobalamin	14.3	11.5
Coenzyme B_{12}	40.9	18.5

Differences in the metabolism of coenzyme B_{12} and cyanocobalamin in rats (*29,30*) and in man (*31*) have been noted which seems to support this interpretation. The transport and tissue distribution were different as shown by preferential uptake of coenzyme B_{12}, especially by liver and kidneys (*29*), and by the dissimilar clearance rates from blood (*29,31*) possibly due to the greater binding capacity of plasma for coenzyme B_{12} than for cyanocobalamin.

3. The calculated period for conversion to coenzyme B_{12} of the hydroxocobalamin and cyanocobalamin retained following massive intramuscular doses should correspond roughly to the period at which "equilibrium" is established and a steady rate of loss is established. Following intramuscular injection of 5,000 μg vitamin B_{12}, there is a very rapid loss over a period of a few days, then less rapid loss during a few months, and thereafter a slow rate of loss (*15*). This is shown diagrammatically in Fig. 1. About 10% (500 μg) of hydroxocobalamin and about 5% (250 μg) of cyanocobalamin are retained. From the respective conversion rate of about 2–5 μg/day and 0.7–1.4 μg/day, the retained analogs will have been largely converted by about 150 days, or less if the conversion rate is higher (*25*). As predicted, it is also about this time that a steady rate of loss is established following intravenous doses of 5,000 μg (*15*).

4. When "equilibrium" has been reached following parenteral doses of hydroxocobalamin, cyanocobalamin, and coenzyme B_{12}, the steady rate of loss should be the same. If administered hydroxocobalamin and cyanocobalamin have been converted

to coenzyme B_{12}, then a common form will reach the excretory system.

Consequently, the excretion rates following "equilibrium" should be independent of the form of the administered analog. Excretion rates measured in man up to 488 days postadministration of 5,000-μg vitamin B_{12} showed no significant difference among the analogs (*15*), and similar findings have been reported in rats (*30*).

THE ROUTINE ESTIMATION OF TOTAL-BODY B_{12}

Serum levels of vitamin B_{12} are a factor considered in the diagnosis of pernicious anemia. It is implied that the fraction of total-body B_{12} in serum is the same among individuals. In some circumstances it might be more meaningful to obtain an estimate of the total-body vitamin B_{12}.

It was shown (*16*) that following a tracer dose of labeled vitamin B_{12} the excretion rate was effectively constant from about 8–10 days postadministration when about 95% of the dose was retained. If "equilibrium" between the tracer B_{12} and native B_{12} was established by this time, then the specific activity (μCi/gm) of tissues and fluids would be the same. Thus if a random urine sample is obtained and assayed:

$$\frac{0.95 \times \text{administered dose } (\mu\text{Ci})}{\text{Total-body } B_{12}\ (\mu\text{g})} = \frac{\text{Activity/ml urine } (\mu\text{Ci/ml})}{B_{12} \text{ content/ml urine } (\mu\text{g/ml})}$$

$$\text{Hence: Total-body } B_{12}\ (\mu\text{g}) = 0.95 \times \text{administered dose } (\mu\text{Ci}) \times \frac{B_{12} \text{ content/ml urine } (\mu\text{g/ml})}{\text{Activity/ml urine } (\mu\text{Ci/ml})}$$

This is simply a statement of isotope dilution with a factor of 0.95 to correct approximately for loss of the tracer dose. If a dose identical to that administered or a known fraction of that administered is counted in the same geometry as the urine sample, then the ratio of the counting rates represents the activity content (μCi).

The test would involve administering the tracer dose to the patient intravenously and preparing an identical standard or known fraction. About 1–2 weeks later, the patient provides a urine sample. A known volume of urine is counted in the same volume and geometry as the standard and a further known volume of urine is assayed to give the amount of vitamin B_{12} in each volume (μg B_{12}/ml). The total-body B_{12} can then be estimated from the relationship.

In practice, the test would be simple, avoiding the necessity for complete urine collection; in addition it uses routinely available facilities and permits examination on an outpatient basis. It can be incorporated with measurements of vitamin B_{12} absorption by whole-body monitoring, which is being used increasingly. An obvious potential problem is the fact that only a fraction of the vitamin B_{12} excreted in urine may be assayable by conventional methods such as microbiological assay (*20,22*). Investigations are in progress to assess the test experimentally.

In principle, the test avoids the assumption implied in the measurements of serum B_{12} that the fraction of total-body B_{12} in the serum is the same among individuals. However, it substitutes the assumption of "equilibrium," for which the justification has been submitted here.

CONCLUSIONS

Following administration of a tracer dose of vitamin B_{12}, the simple and common assumption of "equilibrium" and the assumption that the tracer and native B_{12} behave identically from about 1 week postadministration seem to agree reasonably well with available clinical and experimental data. The alternative of kinetic analysis appears to offer no advantage in interpreting B_{12} metabolism and evidently leads to the conclusion that effectively all of the native B_{12} is in a single compartment as implied by the simple assumption of equilibrium. Because computing facilities and mathematical skills are not required in its interpretation, the simple model is not inferior per se and some might even consider this an advantage. Although the model may be an oversimplification, it appears to be an adequate approximation for most practical clinical purposes.

The assumption of "equilibrium" can be extended to the metabolism of B_{12} analogs. A simple interpretation that hydroxocobalamin and, to a lesser extent, unhydrolysed coenzyme B_{12} and cyanocobalamin are the principal dietary forms and that these analogs are converted to, stored, utilized, and, following "equilibrium," pass to the excretory system in a common form possibly as coenzyme B_{12} seems to explain much of the relevant published data. The present hypothesis does not consider the absorption mechanism of vitamin B_{12}, but it may be capable of extension to this complicated aspect.

A method of estimating total-body B_{12} is proposed based on the assumption of "equilibrium," which involves only the measurement of the specific activity of a casual urine sample about 1–2 weeks postadministration of a labeled tracer dose. Complete urine collection is not required nor is sophisticated equipment needed. The test can be made on an outpatient basis using routinely available techniques. It is currently being evaluated experimentally.

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Validity of the Assumption of Tracer Equilibrium with respect to the Excretion of Vitamin B_{12}

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ABSTRACT. Evidence apparently refuting the assumption of tracer equilibrium shortly after administration of vitamin B_{12} has been critically examined. It is fundamentally impossible to demonstrate differences between the excretion rate of labelled B_{12} and unlabelled B_{12} in the studies described. The apparent changes in the excretion rate of B_{12} are shown to be due to the pooling of data from several patients. The excretion data from whole-body monitoring of individual patients can be adequately described by a single exponential term, if the data from the first few days after administration are not considered. For a single pool system, the assumption of tracer equilibrium is justified. It is shown that excretion rates, calculated on the assumption of tracer equilibrium, agree with those estimated from independent clinical findings based on the time required to develop symptoms of hypovitaminosis following total gastrectomy. The data which dispute the assumption lead to excretion rates which are almost certainly underestimates.

1. Introduction

Controversy has arisen over the common assumption that the excretion rate of a tracer dose of vitamin B_{12} effectively equals that of natural B_{12} in man. Its validity is disputed, notably by Reizenstein, Cronkite and Robertson (1962), Reizenstein, Matthews and Ek (1964 and 1966), Reizenstein (1966) and Heinrich (1964), who suggest that the tracer and natural B_{12} excretion are not the same even after 3 years and that the tracer excretion rate does not become constant until at least 250 days after administration. For the reasons presented here we believe that the contrary evidence is itself controversial.

2. The apparent difference in excretion rates of labelled and 'cold' B_{12}

The only direct experimental measurements reporting an apparent difference between the rates of excretion of labelled and 'cold' B_{12} are evidently those of Reizenstein *et al.* (1962). The data apparently show that the excretion rate of labelled B_{12} (0·1% per day) is greater than that of 'cold' B_{12} (0·03% per day) and Reizenstein *et al.* (1962) conclude that, therefore, 'equilibrium is not reached within the time studied.'

However, examination of the methods used (Reizenstein 1959 and Reizenstein *et al.* 1962) shows that similar quantities of vitamin B_{12} (about 1 μg) were

administered to both series of subjects, who were classified as normal or as having irrelevant diseases. Any perturbation of the steady state resulting from the administered dose of B_{12} can be due only to the presence of additional vitamin, and whether it is labelled or unlabelled is irrelevant in this respect. As there is, apparently, no evidence to suggest that vitamin B_{12} is broken down and the label subsequently lost, whatever the effect of a tracer dose might be on the rate of excretion of B_{12} it should be identical for both series of patients. It should be fundamentally impossible, therefore, to demonstrate a difference in the rates of loss of labelled and non-labelled B_{12} in these particular experiments. As the estimation of the excretion rate of 'cold B_{12}' also involved the administration of labelled B_{12}, and the subsequent assumption that equilibrium was established in about 11 days, the use of this value to argue that equilibrium had not been reached even at 3 years post-administration seems difficult to justify.

3. The apparent changes in excretion rate with time

Reizenstein *et al.* (1964), (1966) and Heinrich (1964) pooled data for several patients and found it necessary to use at least three exponential terms to describe the resultant excretion pattern. They conclude that, as a steady rate of loss is not reached until some time (250 days according to Heinrich (1964)) after administration, equilibrium is not established until that time.

In fact, it is inevitable that a changing rate of excretion is obtained when using pooled data unless the excretion rate for each patient is identical! For example, if the rate of loss for each of three patients can be described by a single exponential term ($e^{-\lambda_1 t}$, $e^{-\lambda_2 t}$, and $e^{-\lambda_3 t}$ respectively) then a full description of the resultant sum curve must presumably include three similar exponents, and be of the form $y = ae^{-\lambda_1 t} + be^{-\lambda_2 t} + ce^{-\lambda_3 t}$. The changing rate of excretion for the pooled data will, therefore, result whether equilibrium is established or not. Only by considering the results for each individual patient can conclusions be drawn concerning changes, or otherwise, of the excretion rate with time.

The retention of labelled vitamin B_{12} has been measured using whole-body monitors by Heyssel and co-workers (1964 and 1966) at tracer levels of dosage, and by Boddy and Adams (1968 b) at tracer and therapeutic (5000 μg B_{12}) dosage levels. These data are treated separately for each patient. Both groups of authors find that excretion following tracer doses can be adequately described by a single exponential term shortly after administration. At the therapeutic level a steady excretion rate is obtained within about one month after administration. Similar conclusions can be drawn from the whole-body monitoring data of Reizenstein and co-workers (1961 and 1962) and of Cohn, Lippincott, Cronkite and Reizenstein (1962) which present results for individual patients.

Reizenstein (1966) indicates that only for single pool systems is the assumption of isotopic equilibrium justified and that such a system and assumption are not valid for vitamin B_{12} metabolism. In contradiction, although Reizenstein *et al.* (1966) initially assume a model of three pools to describe B_{12}

metabolism, they conclude that about 99·3% of the total body B_{12} is, in fact, in a single pool. This evidence, bearing in mind experimental errors, seems to support the conclusion that, for all practical purposes, B_{12} metabolism is essentially a single pool system and, consequently, that the assumption of isotopic equilibrium is justified rather than being evidence to the contrary.

4. The excretion rate of vitamin B_{12} and its probable range

It is assumed that a 'steady state' or equilibrium between tracer and body B_{12} has been reached when excretion can be described by a final single exponential term and this final exponent represents the 'steady-state' excretion rate. Conversely, if subjects with apparently normal B_{12} metabolism undergo total gastrectomy then, since they were in the 'steady-state', subsequent loss of B_{12}, which is not being replenished by dietary absorption, can also be described by a single exponential term.

The development of the haematological and or neurological symptoms of B_{12} hypovitaminosis has been observed in patients from 1 to 14 years after total gastrectomy (Mollin and Ross 1953, Kelly, MacLean, Perry and Wangesteen 1954, Paulson and Harvey 1954, Harvey 1956, Welbourn, Nelson and Zacharias 1958, MacLean and Sundberg 1956, Marchal, Duhamel and Dreyfus 1961, Cox, Williams and Jones 1963, Gol'dberg, Gol'dberg, Lavroka, Polkovnikova, Teterna, Kirikuenko, Golosov, Tinakin and Chernova 1963) with a mean interval of just over 4 years. The normal range of whole-body content of vitamin B_{12} is in the region of 2000–5000 μg (Grasbeck 1959, Adams 1962). If the whole-body content in haematological relapse is reduced to less than 10%, and probably to less than 1% of the original stores as suggested by study of tissues from untreated cases (Haussman 1951, Drouet, Wolff, Karlin-Weissman and Rauber 1951, Girdwood 1952, 1961, Heinrich and Lahann 1954, Swendseid, Hvolboll, Schick and Halsted 1957, Ross and Mollin 1957, Nelson and Doctor 1958, Adams 1962), then the probable range of the excretion rates can be calculated. Assuming simple exponential loss and using the equation $D = D_0 e^{-\lambda t}$, the rate of loss for depletion to 10% and to 1% of the initial body content ranges from 0·63 to 0·045% per day, and from 1·26 to 0·09% per day respectively. The corresponding range for the mean depletion time of about 4·5 years is from 0·28 to 0·14% per day. An excretion rate as high as 26% per day would be observed only when hypovitaminosis occurred within one year at a depletion level of 1%, and an excretion rate as low as 0·045% per day only when depletion to 10% requires 14 years. Excluding these limiting cases, the most probable range may be from about 0·63 to 0·09% per day.

The final excretion rates observed by a number of workers are summarized in table 1 with a note on the measurement techniques. All of the techniques, directly or indirectly, involved the use of labelled B_{12}. With the exception of three reports, all of these data lie within the probable range of excretion rate (0·09–0·63% per day) predicted from the independent clinical findings. It seems hardly co-incidental that the three values significantly lower than this range are those principally cited as evidence disputing the assumption of

Table 1. Final rates of loss of vitamin B_{12}

Author(s)	Technique	Final rates of loss (per cent per day)
Reizenstein (1959 a and b)	Faecal monitoring-tracer	0·09–0·22
Reizenstein *et al.* (1962)	W.B.M.—tracer	0·10
Reizenstein *et al.* (1961)	W.B.M.—tracer	0·17
Cohn *et al.* (1962)	W.B.M.—tracer	0·17
Bozian *et al.* (1963)	W.B.M.—tracer	0·09–0·23
Heyssel *et al.* (1966)	W.B.M.—tracer	0·09–0·17
Boddy and Adams (1967 a)	W.B.M.—5000 μg labelled	0·09–0·19
Boddy and Adams (1967 b)	W.B.M.—tracer	0·08–0·26
Grasbeck (1959)	Faecal and urine monitoring—tracer	0·28
Cronkite *et al.* (1959)	Faecal and urine monitoring—tracer	0·23
Schloesser *et al.* (1958)	Liver monitoring—labelled	0·16–0·21
Glass (1958)	Liver monitoring—labelled	0·08–0·46
Reizenstein (1959 a) and Reizenstein *et al.* (1962)	Non-labelled B_{12}—microbiological and using tracer B_{12}	0·03
Reizenstein *et al.* (1964 and 1966)	Kinetic analysis by computer	0·036
Heinrich (1964)	W.B.M.—tracer	0·051

metabolic equilibrium with respect to excretion. These latter data were also those obtained by 'pooling' results from several patients.

Heinrich (1964) incorrectly calculates a depletion time (to the 10% level) of 4·8 years using his estimate of 0·051% per day for the excretion rate. In doing so, however, he assumes a linear relationship between retention and time, whereas, using the exponential relationship, the depletion time would be longer than 12 years. As Heinrich (1964) accepts an upper limit of about 8 years for depletion time, the value of 0·051% per day would appear to be an underestimate. The corresponding depletion time for the data of Reizenstein *et al.* (1966) (excretion rate of 0·036% per day) is even longer, about 18 years.

The data which assume that equilibrium is established shortly after tracer administration undoubtedly yield excretion rates in better agreement with the clinical observations of depletion time than the data which apparently refute this assumption.

Résumé

La validité de l'hypothèse de l'équilibre de traceurs en ce qui concerne l'excrétion de vitamine B_{12}

On a examiné en critique les faits qui réfutent apparemment l'hypothèse de l'équilibre de traceurs bientôt après l'administration de la vitamine B_{12}. Il est impossible fondamentalement de démontrer des différences entre la vitesse d'excrétion de la vitamine B_{12} marquée et celle de la vitamine non-marquée. On prouve que les variations apparentes de la vitesse d'excrétion de B_{12} sont causées par la mise de données provenant de quelques patients dans une masse commune. Les données de l'excrétion, obtenues par le contrôle du corps entier des patients individuels,

peuvent être décrites d'une manière adéquate par un seul terme exponentiel, si l'on ignore les données relatives aux quelques premiers jours après l'administration. L'hypothèse de l'équilibre des traceurs est justifiée pour un système comprenant une seule masse commune.

On prouve que les vitesses d'excrétion, calculées sur la base de l'hypothèse de l'équilibre des traceurs, sont d'accord avec celles évaluées des résultats cliniques indépendants, basés sur le temps nécessaires pour le développement de symptômes d'hypovitaminose qui apparaissent après une gastrectomie totale. Les données réfutant cette hypothèse conduisent aux vitesses d'excrétion, qui sont presque sans doute infériorisées.

Zusammenfassung

Gültigkeit der Annahme des Tracer-Gleichgewichts in Bezug auf die Ausscheidung von Vitamin B_{12}

Das faktische Material, welches die Annahme des Tracer-Gleichgewichts kurz nach der Verabreichung von Vitamin-B_{12} widerlegt, ist kritisch untersucht worden. Es ist grundsatzlich unmöglich, die Unterschiede zwischen der Ausscheidungsrate von markiertem und nonmarkiertem B_{12} zu beweisen. Es wird gezeigt, dass die scheinbaren Veränderungen in der Ausscheidungsrate von B_{12} darauf zurückzuführen sind, dass man die von mehreren Patienten stammenden Resultate vereinigt. Die Ausscheidungsresultate, die man aus der Ganzkörperüberwachung individueller Patienten erhält, können hinreichend mit Hilfe eines einzigen exponentiellen Terms beschrieben werden, sofern man die sich auf wenige erste Tage nach Verabreichung beziehenden Daten vernachlässigt. Für ein Ein-"Pool"-System ist die Annahme des Tracergleichgewichts gerechtfertigt.

Es wird gezeigt, dass die aus der Annahme des Tracergleichgewichts berechneten Ausscheidungsraten mit denen übereinstimmen, welche auf grund unabhängiger klinischer Befunde berechnet wurden, die sich auf die Zeit stützen, welche benötigt wird, um Hypovitaminose-Symptome nach totaler Magenresektion zu entwickeln. Die diese Annahme vernemenden Daten führen zu Ausscheidungsraten, die fast sicher Unterschätzungen darstellen.

Резюме

Обоснованность предположения равновесия меченых атомов при выделении витамина B_{12}

Факты, повидимому опровергающие предположение о существовании равновесия меченых атомов вскоре после подачи витамина B_{12}, были подвергнуты критическому анализу. Абсолютно невозможно наглядно показать разницу между скоростью выделения меченого и немеченого B_{12}. Показано, что кажущиеся разницы между скоростями выделения B_{12} вызваны объединением результатов, полученных для нескольких пациентов. Результаты выделения, получаемые на основании контроля активности всего тела индивидуальных пациентов, могут быть соответственно описаны при помощи одного показательного выражения, если отбросить данные за несколько первых суток после подачи. В случае единой совокупной системы находит оправдание предположение равновесия меченых атомов.

Показано, что скорости выделения, расчитанные при допущении равновесия меченых атомов, находятся в согласии со скоростями, подсчитанными приблизительно из независимых клинических данных, основывающихся на времени, необходимом для развития симптомов гиповитаминоза после полной гастрэктомии. Данные, полученные при оспаривании этого предположения, дают скорости выделения, которые почти несомненно являются занижениями.

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The Hydrolysis of Vitamin B_{12}. Studies with Model Amides

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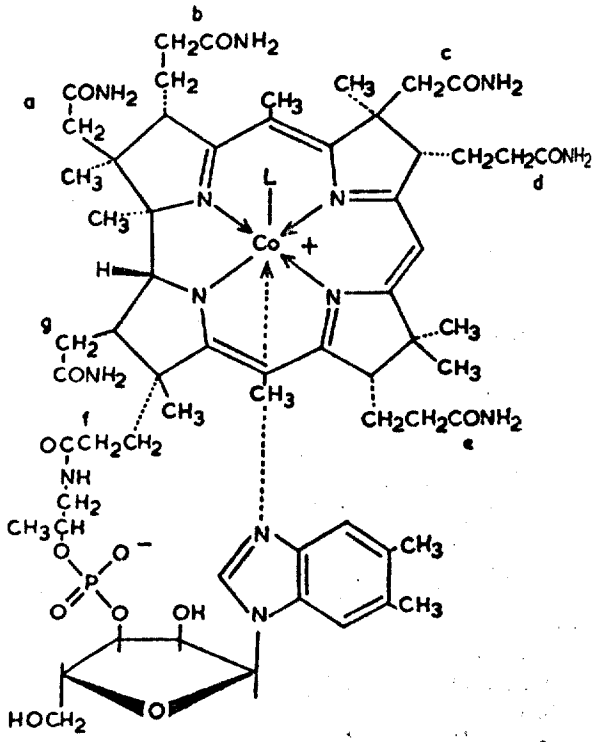
The suitability of certain cyclopentylacylamides as models for the steric situations of the amide groups in vitamin B_{12} is discussed. The syntheses of (1-methylcyclopentyl)acetamide, β-(1-methylcyclopentyl)propionamide, and β-(trans-2,2,3-trimethylcyclopentyl)propionamide are described, and the rates of hydrolysis of these and other model amides in aqueous hydrochloric acid-dioxane at 50° are recorded. Aquocobalamin has been hydrolyzed under identical conditions; comparison of these results with the model kinetics supports the earlier suggestion that steric factors have an important influence in the hydrolysis of vitamin B_{12}.

Introduction

The seven amide functions, two phosphate ester linkages, and ribosylamine group of vitamin B_{12} (I) lead to a complex fragmentation pattern when the vitamin is hydrolyzed.[1] The ribose benziminazole bond, in fact, resists cleavage except under the most vigorous conditions, while methods which are fairly specific for the hydrolysis of the aminopropanol phosphate linkage (leading to cobinamide) have been developed.[1,2] The present concern is the hydrolysis of the amide groups, a process which has been followed in detail[1] by the electrophoresis of the red acidic products. From this work it emerged that a considerable variation existed in the ease of hydrolysis of the amide groups: under mild acidic conditions (*e.g.*, 0.1 *N* HCl, room temperature, several days) three amide groups were cleaved to give mixtures containing three monobasic acids, three dibasic acids, and one tribasic acid, all of which retained the nucleotide. At the other extreme were two amide groups the hydrolysis of which required much more vigorous conditions (2 *N* HCl, 100°, 4 hr.).

A consideration of structure I suggests two major



I, L = CN, vitamin B_{12} (cyanocobalamin)
Ia, L = H_2O, aquocobalamin

(1) (a) J. B. Armitage, J. R. Cannon, A. W. Johnson, L. F. J. Parker, E. Lester Smith, W. H. Stafford, and A. R. Todd, *J. Chem. Soc.*, 3849 (1953); (b) for a review see R. Bonnett, *Chem. Rev.*, 63, 573 (1963).
(2) W. Friedrich and K. Bernhauer, *Z. Naturforsch.*, 9b, 685 (1954).

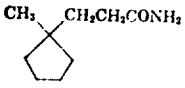
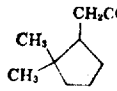
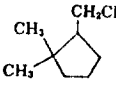
effects which could be responsible for variation in the rate of amide hydrolysis. An enhancement in rate might result from the participation of other structural features in the reaction. Among the groups which might participate are (i) the π-system of the corrin nucleus (*cf.* the anchimerically assisted solvolysis of 2-(Δ^3-cyclopentenyl)ethyl tosylate[3]); (ii) the cobalt atom, insofar as it can coordinate weakly with an amidic nitrogen and thus activate the adjacent carbonyl group (models suggest that neither of these processes is likely to be favored; they both require the acylamide groups to be bent back toward the center of the molecule, and although this may occur under certain circumstances, *e.g.*,[4] acetamide c in crystalline air-dried vitamin B_{12}, it is doubtful whether a significant interaction could arise without considerable steric strain. As far as coordination with the metal is concerned, this may be reasonably considered only for the longer chains (propionamides); the propionamides all lie on the same side of the macrocycle as the nucleotide, so that the observation[5] that the displacement of the latter has not occurred at an acid concentration (0.15 *N*) which cleaves the most labile amides argues against this mechanism.); (iii) other oxygenated functions; for example, the phosphate group might assist the hydrolysis of the propionamide groups, especially amides e and f.[6]

Additionally or alternatively the amide hydrolysis could be controlled by steric factors. Experiments with alkanoic acid amides[7] have shown that β-substitution causes a pronounced decrease in the rate of hydrolysis, while γ-substitution has much less effect. On this basis it has been suggested that the monocarboxylic acids obtained from the vitamin are propionic acid derivatives.[8] Further evidence for this conclusion is provided by the present study of selected model amides.

Selection of Model Amides. The extension of such generalizations as the "rule of six"[9] to the present case offers certain difficulties. These arise largely because the "rule" has been evolved primarily on the behavior of open-chain compounds, because few appropriate cyclic compounds have been studied quantitatively, and because it is difficult to estimate *a priori* what the steric effect of a ring position will be. Newman[9] has used the term "effective six number" (the number of atoms in the 6-position capable of yielding a coiled structure) in recognition of this difficulty. While the effect of ring size and strain on reaction at a ring atom has been extensively studied[10] relatively little is known, in quantitative terms, about substitution reactions in an alkyl chain attached to an alicyclic system.[11] Examples of reaction at a trigonal carbon atom (which are of chief interest here) are few. The effects of ring size on the rate of saponification of ethyl cycloalkylcarboxylates[12] and on the solvolysis of cycloalkylmethyl acetates[13] have been reported. In spite of its bearing on the chemistry of the monoterpenes and the naphthenic acids, the effect of ring substituents on the rate of solvolysis of side-chain carboxylic acid derivatives does not seem to have been evaluated. Since it is desired to examine this effect in relation to vitamin B_{12} hydrolysis, suitable model amides have been sought.

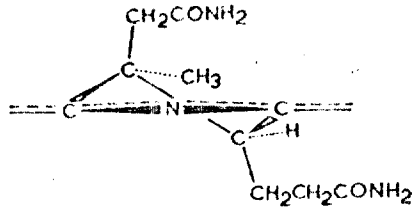
The model systems were selected to approximate the steric situations at the periphery of the macrocycle, but not to represent electronic effects of the unsaturated nucleus. The cyclopentane ring was taken as the basic system; while it has certain limitations (see below) it does fairly represent the tetrahedral arrangement at the β-positions, and with a suitable choice of substituents satisfactory models for individual β-positions can be obtained; see compounds II–V (Table I). In addition,

Table I. Model Amides

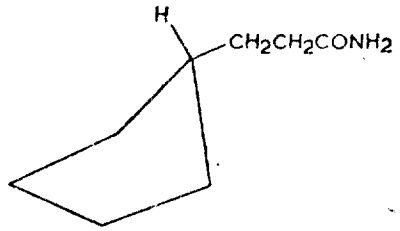
Compd.	Structural formula	Model for position
II		18
III		2, 7
IV		3, 8, 13
V		17
VI		18
VII		3, 8, 13

models (such as VI and VII) which would allow the evaluation of the effect of geminal alkylation at the neighboring carbon atom were desirable. These simple models can scarcely be expected to represent the conformational detail found in vitamin B_{12}; thus,[4] ring B in the crystalline, air-dried vitamin has a conformation represented by VIII, whereas the model for, say, position 8 might be expected somewhat to favor conformation IX (C_s form). It is of interest that in the crystalline vitamin rings A, B, and C adopt the conformation in which two bulky groups are in the quasi-

(3) P. D. Bartlett and S. Bank, *J. Am. Chem. Soc.*, 83, 2591 (1961).
(4) D. C. Hodgkin, J. Lindsay, R. A. Sparks, K. N. Trueblood, and J. G. White, *Proc. Roy. Soc.* (London), A266, 494 (1962).
(5) P. George, D. H. Irvine, and S. C. Glauser, *Ann. N. Y. Acad. Sci.*, 88, 393 (1960).
(6) K. Bernhauer, O. Müller, and F. Wagner in "Vitamin B_{12} und Intrinsic Faktor," H. C. Heinrich, Ed., Second Symposium, Ferdinand Enke Verlag, Stuttgart, 1962, p. 43; C. Zioudrou and G. L. Schmir, *J. Am. Chem. Soc.*, 85, 3258 (1963).
(7) J. Cason and H. J. Wolfhagen, *J. Org. Chem.*, 14, 155 (1949); J. Cason, C. Gastaldo, D. L. Glusker, J. Allinger, and L. B. Ash, *ibid.*, 18, 1129 (1953).
(8) R. Bonnett, J. R. Cannon, V. M. Clark, A. W. Johnson, L. F. J. Parker, E. L. Smith, and A. Todd, *J. Chem. Soc.*, 1158 (1957).
(9) M. S. Newman, *J. Am. Chem. Soc.*, 72, 4783 (1950).
(10) H. C. Brown, *J. Chem. Soc.*, 1248 (1956); C. H. Collins and G. S. Hammond, *J. Org. Chem.*, 25, 698 (1960); T. C. Bruice and U. K. Pandit, *J. Am. Chem. Soc.*, 82, 5858 (1960); O. H. Wheeler and M. A. Almeida, *J. Org. Chem.*, 27, 4448 (1962); H. C. Brown and F. J. Chloupek, *J. Am. Chem. Soc.*, 85, 2322 (1963).
(11) E. E. Royals and A. H. Neal, *J. Org. Chem.*, 21, 1448 (1956); C. F. Wilcox and S. S. Chibber, *ibid.*, 27, 2332 (1962); G. S. Skinner and F. P. F. Florentine, *J. Am. Chem. Soc.*, 76, 3200 (1954).
(12) O. H. Wheeler, O. Chao, and J. R. Sanchez-Caldas, *J. Org. Chem.*, 26, 2505 (1961).
(13) S. Sarel, L. Tsai, and M. S. Newman, *J. Am. Chem. Soc.*, 78, 5420 (1956).



VIII



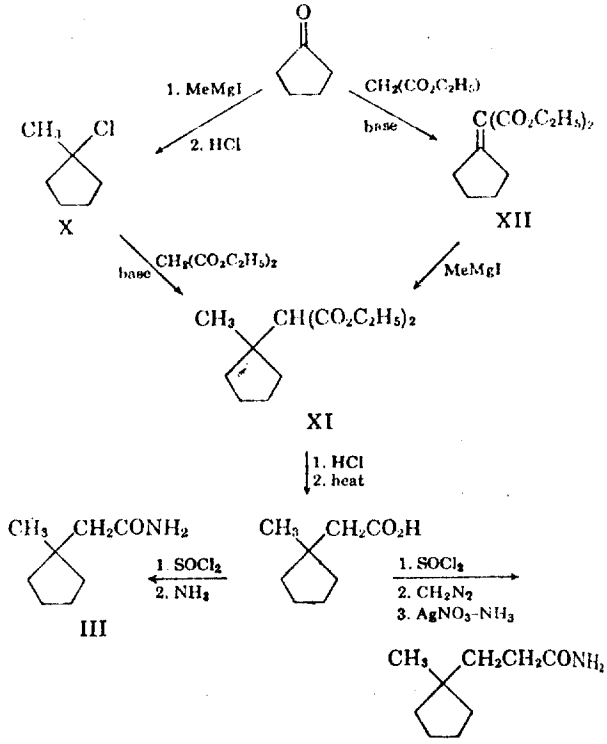
IX

axial situation, an arrangement which presumably accommodates the steric effect of the methyl substituents at C-5 and C-15. However certain changes in the conformations of the β-positions have been observed in other corrinoids (*e.g.*, cobyric acid[14]), and even in the "wet" vitamin B_{12} crystal the situation is slightly changed.[15] It seems possible that in solutions of the vitamin the β-positions are somewhat flexible. Moreover, the energies involved in the interconversion of conformers of cyclopentanes[16] are expected to be smaller than those observed in the more familiar cyclohexane series, and since the projected hydrolyses are to be carried out at an elevated temperature, and the reacting group is in any case not directly attached to the ring system, it is considered that the conformational inequalities of the model compounds, both among themselves and with respect to the vitamin, are not likely to be significant compared with the gross steric effect of substitution.

Synthesis of Model Amides. Cyclopentylacetamide and cyclopentylpropionamide were readily available. The 1-methyl derivatives of these compounds were unknown and were synthesized from cyclopentanone, as shown in Chart I.

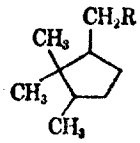
Two routes to diethyl (1-methylcyclopentyl)malonate (XI) were considered. The first involved attempted nucleophilic substitution of the tertiary halide X, where elimination would be expected to be favored. However, under suitable reaction conditions (extended reaction period at room temperature) the substitution proceeded to give the malonate XI in a yield which though low (13%) was acceptable in view of the availability of the starting materials. Understandably, there appear to be few examples of this type of preparation recorded; Dox and Bywater[17] report the reaction of *t*-butyl bromide and diethyl malonate to give the substitution product in 6.4% yield. The alternative route to XI involved the conjugate addition of methylmagnesium iodide to the unsaturated ester XII. With diethyl isopropylidenemalonate, this type of addition appears to proceed without difficulty,[18] but in the present case the product, presumably contaminated with 1,2-adducts, had to be purified by gas–liquid chromatography.

Chart I. Synthesis of (1-Methylcyclopentyl)acylamides



The remaining steps (Chart I) were straightforward. The n.m.r. spectrum of (1-methylcyclopentyl)acetamide (III) confirmed that rearrangement had not occurred during the reaction sequence; both the methyl signal (τ 8.91) and the deshielded methylene signal (τ 7.82) were sharp singlets.

In elaborating compounds of type VI and VII it was convenient to use camphor as the starting material, and thus employ α-campholanamide (XIII) as the model. It was considered that the 3-methyl substituent in XIII would be unlikely to interfere significantly with kinetic observations. *trans*-α-Campholanonitrile (XV) is reported to be available in one step by the thermal cleavage of camphorimine in the presence of oxygen.[19] This interesting reaction is clearly quite complex; vapor phase chromatography shows that the more volatile fraction of the product consists of four components, *viz.* (i) camphor; (ii) a liquid, not observed by previous workers, the infrared spectrum of which closely resembles that of α-campholenonitrile; (iii) *trans*-α-campholanonitrile[19,20] (the major constituent); and (iv) a liquid, the infrared spectrum of which suggests a ketonitrile formulation (*cf.* the



XIII, R = $CONH_2$
XIV, R = COOH
XV, R = CN
XVI, R = CH_2CONH_2

(14) D. C. Hodgkin, private communication.
(15) C. Brink-Shoemaker, D. W. J. Cruickshank, D. C. Hodgkin, M. J. Kamper, and D. Pilling, *Proc. Roy. Soc.* (London), A278, 1 (1964).
(16) K. S. Pitzer and W. E. Donath, *J. Am. Chem. Soc.*, 81, 3213 (1959).
(17) A. W. Dox and W. G. Bywater, *ibid.*, 58, 731 (1936).
(18) G. A. R. Kon and E. A. Speight, *J. Chem. Soc.*, 2727 (1926); S. Wideqvist, *Chem. Abstr.*, 41, 1615 (1947).
(19) F. Mahla and F. Tiemann, *Ber.*, 33, 1929 (1900).
(20) P. Lipp, *ibid.*, 55, 1883 (1922).

isopropyl-6-keto heptanonitrile reported by Mahla and Tiemann[16]). The camphor presumably arises by hydrolysis (either during the reaction—camphorimine is rather hygroscopic—or during work-up). The pathways by which the other components arise deserve further study, but, for the present purpose, this reaction, followed by preparative gas–liquid chromatography, proved to be a convenient route to α-campholanonitrile (XV) which on further elaboration gave both the acetamide XIII and the propionamide XVI derivatives required for the kinetic work.

Rate Measurements. The rates of hydrolysis of the six cyclopentylacylamides were observed under acidic (1.5 *N* HCl, aqueous dioxane, 50°) and basic (potassium hydroxide in propanol, at reflux temperature) conditions. In addition the hydrolyses of some open-chain analogs (XVII and XVIII) and of aquocobalamin (Ia) were examined under the acidic conditions. Aquocobalamin (Ia) rather than cyanocobalamin (I) was chosen for this work to avoid the interference of cyanide hydrolysis.

Experimental[21]

Cyclopentylacetamide, prepared from the corresponding acid in the usual way (thionyl chloride, ammonia), was recrystallized from benzene as white plates, m.p. 150–151° (lit.[22] m.p. 150°).

Anal. Calcd. for $C_7H_{13}NO$: C, 66.10; H, 10.30; N, 11.01. Found: C, 66.24; H, 10.30; N, 11.09.

β-Cyclopentylpropionamide similarly obtained had m.p. 124–125.5° (lit.[23] m.p. 122–123°).

Anal. Calcd. for $C_8H_{15}NO$: C, 68.04; H, 10.71; N, 9.92. Found: C, 68.06; H, 10.63; N, 9.90.

Diethyl (1-Methylcyclopentyl)malonate (XI). A. A mixture of 1-methylcyclopentyl chloride (152.5 g.) and diethyl malonate (206 g.) was added to an ethanolic solution of sodium ethoxide (33.6 g. of sodium in 600 ml. of anhydrous ethanol). The resulting solid mass was warmed and broken up, and then stirred for 5.5 days at room temperature. The volume of the liquid was reduced to ~250 ml. (rotary evaporator), water (400 ml.) was added, and the organic layer was separated. It was washed with salt solution, and, combined with an ethereal extract of the aqueous phases, was dried (Na_2SO_4) and distilled to give 40.8 g. (13%) of diethyl (1-methylcyclopentyl)malonate, b.p. 88.5–89.5° at 1.3 mm., $n^{20}D$ 1.4500.

Anal. Calcd. for $C_{13}H_{22}O_4$: C, 64.44; H, 9.15. Found: C, 64.67; H, 9.10.

Diethyl malonate (66%) was recovered.

B. To methylmagnesium iodide (from 8.6 g. of methyl iodide) cooled and stirred in ether (30 ml.) was gradually added diethyl cyclopentylidenemalonate[18] (14 g.) in ether (30 ml.). The mixture was refluxed for 10 min. and worked up[18] to yield 7.5 g. of a liquid, b.p. 98–106° at 2.3 mm. Redistillation gave 5.2 g., b.p. 98–100° at 2.2 mm., the infrared spectrum (film) of which showed an –OH band. Gas–liquid chromatography (Aerograph A-90-P, Apiezon J at 200°, helium carrier) separated three components of retention volumes 180 ml. (one part), 440 ml. (three parts), and 930 ml. (ten parts). The last component was collected. It had an infrared spectrum essentially identical with that of the product described under A above.

(1-Methylcyclopentyl)acetic Acid. Diethyl (1-methylcyclopentyl)malonate (9.17 g.) was refluxed (steam bath) with 10% ethanolic potassium hydroxide (40 ml.) for 13 hr. The cold solution deposited hygroscopic plates which were separated and dissolved in water (50 ml.). The acidified (dilute sulfuric acid) solution was continuously extracted with ether (3.5 hr.). The ether was removed and the residue was recrystallized from benzene to give 4.4 g. of (1-methylcyclopentyl)-malonic acid. A further 0.67 g. was obtained by making the alcoholic hydrolysate 30% in potassium hydroxide and refluxing for an additional 10 hr., yielding 5.07 g. (72%), m.p. 136–137°.

(1-Methylcyclopentyl)malonic acid (5 g.) was heated in an upright short-path distillation apparatus for 6 hr. at 160°; the evolution of carbon dioxide appeared to be complete in ~2.5 hr. The dark liquid was distilled to give 3.3 g. (86%) of (1-methylcyclopentyl)-acetic acid as a sour-smelling liquid, b.p. 74° at 0.22 mm.

Anal. Calcd. for $C_8H_{14}O_2$: C, 67.57; H, 9.93. Found: C, 67.29; H, 9.79.

(1-Methylcyclopentyl)acetamide (III). This was prepared from the acid chloride (b.p. 70–72° at 12 mm.) and formed white platelets, m.p. 96–97.5°, from benzene; n.m.r. spectrum (Varian A60, $CDCl_3$, internal standard tetramethylsilane): τ ~4.0 (broad, NH), 7.82 (singlet, $-CH_2CO-$), ~8.4 (multiplet, ring CH_2); and 8.91 (singlet, CH_3-).

Anal. Calcd. for $C_8H_{15}NO$: C, 68.04; H, 10.71; N, 9.92. Found: C, 68.14; H, 10.87; N, 10.18.

β-(1-Methylcyclopentyl)propionamide (V). (1-Methylcyclopentyl)acetyl chloride (5.8 g.) in ether (70 ml.) was added to an excess of a cold ethereal solution of diazomethane. The solution was allowed to warm up to room temperature. Evaporation of the ether gave ~5 g. of the crude diazo ketone which was dissolved in dioxane (150 ml.). Aqueous ammonia (*d* 0.88, 50 ml.) and 10% aqueous silver nitrate (10 ml.) were added. The mixture was heated for 1 hr. at 60–70° and, after the addition of more water (200 ml.), for an additional hour on the steam bath. The resulting suspension was treated with charcoal and filtered; the cold filtrate was saturated with sodium chloride and extracted with ether. Removal of the ether and crystallization (charcoal) from ethyl acetate gave 2.27 g. (42%) of β-(1-methylcyclopentyl)propionamide, m.p. 105–110°; recrystallized from benzene, m.p. 109–110°.

Anal. Calcd. for $C_9H_{17}NO$: C, 69.63; H, 11.04; N, 9.02. Found: C, 69.60; H, 10.97; N, 9.23.

trans-2,2,3-Trimethylcyclopentylacetonitrile (trans-α-Campholanonitrile) (XV).[19] A fine stream of air was bubbled for 10 hr. through molten camphorimine (85 g.) in an oil bath at 110–120°, the exit gases being passed *via* a condenser through a trap containing a small quantity of ethanol. The ethanol solution was concentrated and combined with the reddish brown reaction product, which was washed successively with dilute acid, dilute base, and water, and then steam distilled.

(21) Melting points are uncorrected. Analyses were by A. Bernhardt (Mülheim) and Mrs. Aldridge (Chemistry Dept., University of British Columbia).

(22) W. E. Doering and L. H. Knox, *J. Am. Chem. Soc.*, 78, 4947 (1956).

(23) V. Zikan and M. Semonsky, *Collection Czech. Chem. Commun.*, 24, 1274 (1959).

The distillate (~2.5 l.) was salted and extracted with ether, the extract being dried overnight ($CaSO_4$). Distillation gave two fractions, b.p. 104–118° at 20 mm. (21.7 g.) and b.p. 124–160° at 20 mm. (6 g.).

The experiment was repeated to obtain a total of 92 g. of the lower boiling fraction. This was further fractionated by preparative gas liquid chromatography (Beckman Megachrome, Apiezon J at 215°, helium carrier). Four fractions were obtained: (i) camphor, m.p. 96°, oxime, m.p. 116°, m.m.p. 116–118°, 18% of the lower boiling fraction; (ii) a liquid, ν_{max} 2959 (s), 2237 (w), 1733 (w), and 800 (m) cm.$^{-1}$; 9% of the lower boiling fraction; (iii) *trans*-2,2,3-trimethylcyclopentylacetonitrile, b.p. 110.5° at 6 mm. (lit.[20] for inactive material b.p. 112–117° at 16 mm.), n^{20}D 1.4570 (lit.[19] nD 1.4611) 39.8 g. (69% of lower boiling fraction, 11% over-all); (iv) a liquid, ν_{max} 2985, 2242, 1715, and 1163 cm.$^{-1}$; 1% of lower boiling fraction.

trans-2,2,3-Trimethylcyclopentylacetamide (*trans-α-Campholanamide*) (*XIII*). *trans*-2,2,3-Trimethylcyclopentylacetonitrile (37.8 g.) was refluxed with 30% ethanolic potassium hydroxide for 24 hr. The ethanol was removed and the residue was dissolved in water; this solution was made strongly acidic (dilute sulfuric acid) and extracted with ether. The dried (Na_2SO_4) extract was distilled to give 35.5 g. (83%) of *trans*-2,3,3-trimethylcyclopentylacetic acid, b.p. 149–154° at 6 mm., n^{20}D 1.4628 (lit.[19] b.p. 160° at 22 mm., nD 1.4628). The corresponding amide was prepared in the usual way, yielding colorless plates, m.p. 145.5–146.5°, from benzene (lit.[19] 143° from ethyl acetate).

Anal. Calcd. for $C_{10}H_{19}NO$: C, 70.96; H, 11.32; N, 8.28. Found: C, 70.76; H, 11.26; N, 8.32.

(*trans-2,2,3-Trimethylcyclopentyl*)*propionamide* (*XVI*). This was prepared from *trans*-2,2,3-trimethylcyclopentylacetyl chloride, b.p. 98–100° at 15 mm., in an analogous fashion to the preparation of V above. This gave 24% (based on XIV) of the amide, white lustrous platelets, m.p. 83.5–84°, from benzene petroleum ether (b.p. 60–80°).

Anal. Calcd. for $C_{11}H_{21}NO$: C, 72.08; H, 11.55; N, 7.64. Found: C, 72.09; H, 11.51; N, 7.89.

Other Amides. Isovaleramide (XVII) was obtained from the corresponding acid (thionyl chloride, ammonia) and recrystallized thrice from benzene light petroleum ether (b.p. 60–80°). It was dried *in vacuo* at 50° for 4 hr., m.p. 136–137° (lit.[24] 136–137°).

Anal. Calcd. for $C_5H_{11}NO$: C, 59.37; H, 10.96; N, 13.85. Found: C, 59.62; H, 10.84; N, 13.81.

t-Butylacetamide (XVIII) was prepared and purified in a similar manner, m.p. 130–131° (lit.[25] 132°).

Anal. Calcd. for $C_6H_{13}NO$: C, 62.57; H, 11.38; N, 12.16. Found: C, 62.31; H, 11.31; N, 12.24.

Aquocobalamin, cyanocobalamin, and cobinamide were supplied by Glaxo Research Ltd. through the courtesy of Dr. E. Lester Smith.

Kinetic Measurements. The amide was dissolved in dioxane and the solution was then diluted to 4 volumes with 2 *N* hydrochloric acid (1.5 *N* HCl in 25% aqueous dioxane). An amide concentration of 0.05 *M* was used. The experiments were performed in duplicate in sealed tubes in a thermostatic bath at 50.0 ± 0.05°. At given intervals a tube was removed, cooled, and opened. Five milliliters of the solution was withdrawn and neutralized (*m*-cresol purple) with 1 *N* potassium hydroxide solution. The neutralized solution was buffered to pH 8.5 with borate buffer (0.05 *M*, 15 ml.) in a Markham still.[26,27] Steam distillation was continued for 10 min. and the liberated ammonia was trapped in 2% aqueous boric acid (20 ml.). The ammonia was determined by titration with 0.01 *N* HCl (methyl red–methylene blue mixed indicator). An initial blank was determined in all runs using amide solution which had not been heated to 50°. Titration values for total hydrolyses and for standard ammonium chloride samples were in accord with calculated values.

The hydrolyses of compounds XIII and XVI were accompanied by some separation of the corresponding acids; this did not appreciably alter the observed rate, however.

The hydrolysis of aquocobalamin was carried out in a similar way, except that an inert atmosphere was used. The aquocobalamin was not dried since it has been shown that this may cause slight decomposition.[28] Dioxane (25 ml.) was diluted to 100 ml. with 2 *N* hydrochloric acid and flushed with oxygen-free nitrogen for 0.5 hr. Aquocobalamin (150 mg., 18% hydration, 0.00365 *M*) was dissolved in 25 ml. of this solution and portions were sealed under nitrogen and treated as before. Ammonia was estimated with 0.002 *N* hydrochloric acid. Control experiments indicated that ammonia cobalichrome formation and the liberation of 1-aminopropan-2-ol did not interfere significantly with the determination. Titration values for standard ammonium chloride samples were in accord with the calculated values. The residue from each reaction tube was extracted by the phenol–ether method[1] and examined by paper electrophoresis (pH 6.5 and 10) for corrinoid carboxylic acids.[1] For comparison cobinamide and cyanocobalamin were hydrolyzed under the same conditions, and the progress of hydrolysis was followed by electrophoresis.

Results

Model Amides. All the simple amides gave good straight-line plots of t against log $a/(a - x)$ and the

Table II. Rates of Acid Hydrolysis of Amides in Aqueous Dioxane at 50° (1.5 *N* HCl)

Amide	First-order rate constant $k \times 10^4$ min.$^{-1}$ — First series	Second series	Average
II	13.4	13.3	13.3
IV	36.5	37.4	37.0
III	1.22	1.19	1.2
V	36.5	34.9	35.7
XIII	11.2	11.2	11.2
XVI	30.7	29.9	30.3
XVII[a]	6.65	6.77	6.7
XVIII[b]	0.94	0.96	0.95

[a] $(CH_3)_2CHCH_2CONH_2$. [b] $(CH_3)_3CCH_2CONH_2$.

(24) G. E. Philbrick, *J. Org. Chem.*, 19, 623 (1954).
(25) A. H. Homeyer, F. C. Whitmore, and V. H. Wallingford, *J. Am. Chem. Soc.*, 55, 4209 (1933).
(26) C. L. Wilson and D. W. Wilson, "Comprehensive Analytical Chemistry," Vol. 1B, Elsevier, Amsterdam, 1960, p. 500.
(27) J. M. Brierly, R. R. Sealock, and H. Diehl, *Iowa State Coll. J. Sci.*, 29, 141 (1954).
(28) E. Lester Smith, *Analyst*, 87, 183 (1962).

Table III. Acid Hydrolysis of Aquocobalamin in Aqueous Dioxane at 50° (1.5 *N* HCl)[a]

Time, hr.	NH_3[b] evolved, %	Unchanged B_{12a}	Cobinamide	Monoacid +Nt	Monoacid −Nt	Diacid +Nt	Diacid −Nt	Triacid +Nt	Triacid −Nt	Tetraacid	Pentaacid	Hexaacid	Heptaacid
0.5	10.1	XXX	X	XX		X							
1	14.6	XX	X	XX	X	XX							
2	23.7	XX	X	XX	X	XX	X	X					
3.5	34.4	X	X	XX	X	XX	XX	X	X				
6	44.2	X	..	X	X	XX	XX	XX	XX	X			
11	52.5	...	..	...	...	...	X	XX	XX	XX	X		
26	63.0	...	..	...	...	...	...	...	XX	XXX	XX	X	
53	65.0	...	..	...	...	...	...	...	X	XX	XXX	XX	
120	81.9	...	..	...	...	...	...	...	...	X	XXX	XXX	X
220	92.7	...	..	...	...	...	...	...	...	...	X	XXX	XXX

[a] XXX, major component; XX, moderate component; X, minor component. [b] Assuming 6 moles of NH_3.

Table IV. Acid Hydrolysis of Cyanocobalamin in Aqueous Dioxane at 50° (1.5 *N* HCl)[a,c]

Time, hr.	B_{12}	Cobinamide	Monoacid[b] +Nt	Monoacid[b] −Nt	Diacid[b] +Nt	Diacid[b] −Nt	Triacid, −Nt	Tetraacid
0.5	XXX	XX	XX	X	X			
1	XX	XX	XX	XX	X	X		
2	X	XX	XX	XX	X	XX	X	
3.5	...	X	X	XX	X	XX	XX	X
6	...	...	...	XX	...	XXX	XX	

[a] XXX, major component; XX, moderate component; X, minor component. [b] Polycarboxylic acids containing four or more carboxylic acid groups do not retain the nucleotide in any of the hydrolyses described in this paper. [c] +Nt, nucleotide containing; −Nt, nucleotide free.

hydrolyses were thus, as expected, first order with respect to amide. The rate constants are given in Table II; the deviations in these values, estimated from the worst plot in each graph, were not greater than 4%.

Corrinoid Derivatives. The results of the hydrolysis of aquocobalamin are indicated in Table III. The ammonia evolution was calculated on the basis of the six primary amide groups, it having been shown that 1-amino-2-propanol did not significantly interfere under the conditions used since it is much less steam volatile than is ammonia. The carboxylic acids were examined by paper electrophoresis[1] at pH 6.5 and 10. The amounts of individual fractions in relation to the total pigmented product were estimated visually and are recorded in Table III on a scale which is also used in Tables IV and V, which indicate the progress of hydrolyses of cyanocobalamin and cobinamide under the same conditions. In the last case the monocarboxylic acid fraction was isolated after hydrolysis (0.75 hr. in aqueous 1.5 *N* HCl at 50°) and shown by paper chromatography (butan-2-ol–aqueous ammonia (2:1) containing HCN, Whatman 3MM paper, downward development for 72 hr.) to consist of three components (ratio ~2:1:1) none of which was cobyric acid.

Table V. Acid Hydrolysis of Cobinamide in Aqueous Dioxane at 50° (1.5 *N* HCl)[a]

Time, hr.	Cobinamide	Monoacid	Diacid	Triacid	Tetraacid
0.5	XXX	XX	X		
1	XXX	XXX	XX		
2	XX	XXX	XX	X	
3.5	X	XX	XXX	XX	X
6	...	X	XXX	XXX	X

[a] XXX, major component; XX, moderate component; X, minor component.

Discussion

It is intended to discuss the trends observed in the hydrolysis of the simple amides, and then to relate these, where possible, to the hydrolysis of the vitamin B_{12} derivatives. It is assumed that the mechanism of hydrolysis is the same throughout the model series, *i.e.*, the rate-determining step will be the attack of water on the protonated amide function.[29] It may also be expected that for the models, differences in rate will be due essentially to steric factors. Polar effects in the acid hydrolysis of the related esters are known to be slight[30]; and, in any case, the effect would not be expected to be important here since the substituents occur at the β- or γ-positions, where, as the σ^* values indicate,[30] their polar influence is small.

Model Amides. The following conclusions emerge from the data in Table II.

(i) Cyclopentylacetamides are hydrolyzed more slowly than the corresponding cyclopentylpropionamides. In the examples which have no methyl group at the ring–chain junction (II and XIII compared with IV and XVI, respectively) the ratio of the rates is about 2.7.

(ii) Substitution of a methyl group at the β-position of the acetamide (II → III) results in a pronounced rate decrease by a factor of about eleven; in the corresponding propionamide case the lowering of the rate constant caused by γ-methylation (IV → V) is barely observable. These results have close analogies in the hydrolyses of

(29) M. L. Bender and R. D. Ginger, *J. Am. Chem. Soc.*, 77, 348 (1955).

(30) J. T. Edward and S. C. R. Meacock, *J. Chem. Soc.*, 2000 (1957); R. W. Taft, "Steric Effects in Organic Chemistry," M. S. Newman, Ed., John Wiley and Sons, Inc., New York, N. Y., 1956, p. 556.

open-chain aliphatic amides[7]; certain modifications in detail do appear however. These are presumably associated with the compactness of the ring system and may now be mentioned.

(iii) γ- or δ-substitution has little effect on the hydrolyses of straight chain amides[7] or esters.[30] In the cyclopentylalkanoic acid system studied here, however, a *gem*-dimethyl group adjacent to the acylamide side chain (XIII, XVI) is associated with a small but definite decrease in rate. Presumably, in spite of the puckering possible in the cyclopentane ring, the acylamide function and one of the methyl groups can interact significantly. An effect of the same origin but of much greater magnitude is found in the esterification of the 2,5-dimethylcyclopentanecarboxylic acids.[31] It is of interest that the effect of adjacent substitution in the ring shows up even in the propionamide XVI.

(iv) The rate of hydrolysis of isovaleramide (XVII) is less than that of cyclopentylacetamide (II), while *t*-butylacetamide (XVIII) is hydrolyzed somewhat more slowly than is (1-methylcyclopentyl)acetamide (III). It appears that in this situation the steric effect of the cyclopentyl group is less than that of the isopropyl group; similar behavior has been observed in other systems (Chart II). This is somewhat surprising since the E_s values[30] (hydrolysis of RCOOR′) suggest that the cyclopentyl group (R; $E_s = -0.51$) exerts slightly more steric influence than the isopropyl group (R; $E_s = -0.47$). The differences involved are, however, quite small, and it is concluded that for the chain lengths under observation the steric effect of the cyclopentyl group is of the same order as that of the isopropyl group, but is smaller than that of the diethylcarbinyl group which is the strict open-chain analog of the five-membered ring. Formation of a five-membered ring places the ring members under a constraint, severely restricts the loci of the ring atoms and their immediate substituent atoms, and necessarily diminishes the total steric requirement.

(v) The alkaline hydrolysis of the six cyclopentylacylamides, carried out by the method of Cason and co-workers,[7] gave kinetic data (Table VI) which followed the same pattern as that already discussed. This confirms the importance of the steric effects revealed in the acid hydrolysis of the model amides.

Table VI. Rates of Basic Hydrolysis of Amides in Propanol at 95° (0.5 *N* KOH)

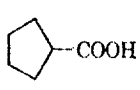
Amide	k_2, l. mole^{-1} hr.$^{-1}$	Amide	k_2, l. mole^{-1} hr.$^{-1}$
II	0.095	V	0.27
IV	0.30	XIII	0.090
III	0.011	XVI	0.27

Amide Hydrolysis in Cyano- and Aquocobalamins. In extensive studies on the hydrolysis of vitamin B_{12}, Todd and his co-workers[3] showed that the vitamin contained three readily hydrolyzed amide groups, while there was evidence for four more amide groups, two of which were rather resistant to hydrolysis. None of the three labile amides was the secondary amide f since the corresponding products retained the nucleotide. The nucleotide–propanolamine moiety could nevertheless be removed at a fairly early stage and was not associated with the two most resistant amide functions. As is apparent from Table III, the acid-catalyzed hydrolysis of aquocobalamin in aqueous dioxane follows the same general course.

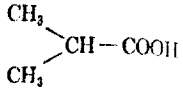
Clearly the model experiments strongly support the earlier suggestion[8] that the monocarboxylic acids are propionamide derivatives. The model propionamide IV (six-number[9] = 3), representing side chains b, d, and e, is hydrolyzed 30 times faster than the methylated acetamide III (total six-number = 9; effective six-number, *i.e.*, not counting ring carbons in six position, = 7) representing the amide groups at a and c, which are therefore considered to be the two groups most resistant to hydrolysis. The acetamide II without the β-methyl group is much less affected; it is hydrolyzed about 10 times faster than the highly hindered amide III and it is presumed, therefore, that amide g, to which it corresponds, is one of the two amides which are hydrolyzed at an intermediate rate. The other is then amide f. The model V shows that γ-methylation at the ring junction has only a slight hindering effect; a

Chart II. Relative Reaction Rates for the Analogous Cyclic and Open Chain Compounds[a]

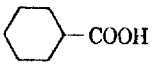
1. Esterification of carboxylic acids at 25° (α-branching)



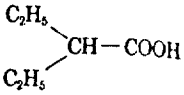
1.0



0.80

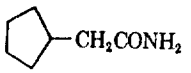


0.41

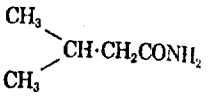


0.016

2. Acidic hydrolysis of amides at 50° (β-branching)

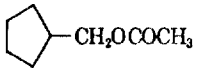


1.0

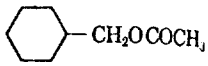


0.5

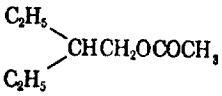
3. Alkaline hydrolysis of alkyl acetates at 20° (γ-branching)



1.0



0.63



0.32

[a] In each series the rate of the specified reaction for the cyclopentyl derivatives is set at unity. [b] G. D. Advani and J. J. Sudborough, *J. Indian Inst. Sci.*, 6, 41 (1923). [c] S. Sarel, L. Tsai, and M. S. Newman, *J. Am. Chem. Soc.*, 78, 5420 (1956).

(31) T. L. Jacobs and W. H. Florsheim, *J. Am. Chem. Soc.*, 72, 261 (1950).

steric effect in the secondary amide function, which is not represented in the models, would also be expected (*cf.* the hindered alkyl acetates[32]). In addition there exists the possibility of an assisted cleavage of amide f; however, this appears to be important only under conditions of high acidity[33] and on the basis of the steric effects alone this amide would be expected to be hydrolyzed somewhat more slowly than the other three propionamides. The similarity of the hydrolysis product patterns for aquocobalamin (Table III) and cobinamide (Table V) suggests that the nucleotide does not have a profound effect on amide hydrolysis under these conditions.

Table VII. Relative Rates of Amide Hydrolysis

Model	Side chains represented	Relative rate of acid hydrolysis
II	g	11
IV	b, d, e	31
III	a, c	1.0
V	(f)	30

A semiquantitative relationship between the model amide hydrolyses and the hydrolysis of aquocobalamin must be somewhat arbitrary, but may be attempted. If the assumption is made that during the first 3.5 hr. only the three most labile amide groups of aquocobalamin are being hydrolyzed, then an apparent first-order rate constant for these groups ("k" = 55×10^{-4} min.$^{-1}$) may be extracted from the hydrolysis data (Table III). This value is to be compared with cyclopentylpropionamide $k = 37 \times 10^{-4}$ min.$^{-1}$; clearly the agreement is reasonable, and is improved by making allowance for the slow generation of ammonia from the other amides. After about 150 hr. essentially only the two most resistant amides remain (Table III). An apparent rate constant "k" = 1.1×10^{-4} min.$^{-1}$ may be obtained from this portion of the hydrolysis, and may be compared with the figure for (1-methylcyclopentyl)acetamide $k = 1.2 \times 10^{-4}$ min.$^{-1}$. Although it is not possible to compare individual rate constants, the similarities thus demonstrated between the extreme models and the extreme portions of the complex hydrolysis do suggest that the models chosen were suitable, and it follows that steric effects are of much importance in the hydrolysis of vitamin B_{12}.

Certain structural consequences may be mentioned briefly.[1b] It is apparent that the steric effects operate so as to reduce the number of isomeric acids which can be isolated; thus under normal hydrolytic conditions the mixture of monocarboxylic acids has three detectable components and not seven. Further support is now available for the view that the "antivitamins" (mono-N-substituted amides derived from the monocarboxylic acids) prepared by L. Smith[34] are propionic acid, and not acetic acid, derivatives. It is also of interest to consider probable structures for the pentacarboxylic acid(s) which can be isolated from vigorous alkaline hydrolysis of the vitamin.[3c] This hydrolysis is complicated by the cyclization of acetamide c to give a fused lactam structure[4b] on ring B so that a hexacarboxylic acid is a product of total hydrolysis. The pentacarboxylic acid presumably has in addition the hindered acetamide residue at a; experiments are planned to elucidate whether or not this acetamide has also cyclized to give a lactam on ring A.

It is not to be concluded, of course, that in hydrolyses of such a complicated system steric effects alone will operate. Thus a comparison of Tables III and IV shows that cyanocobalamin, under the conditions employed, loses the nucleotide more rapidly than does aquocobalamin. This is presumably due to the greater *trans* effect of the cyano ligand[7] in labilizing the base–cobalt linkage; when this is broken chain f can adopt a conformation away from the corrinoid system and is thus more accessible to attacking reagents. Again, it has been found[1] that one of the three labile amide groups is more reactive than the other two. This is most readily observed under very mild conditions (*e.g.*, 0.02 *N* HCl, 20 days at ambient temperature) and might be due to an assisted hydrolysis. The acylamide chains are alternately up and down and may not interact significantly with one another, but participation of the nucleotide in the hydrolysis of amide e might be feasible.[6] Alternatively the distinction could be due to the steric effects of the methyl groups at C-5 and C-15 on the propionamides at the adjacent β-positions (this would result in propionamide d being the most labile). A third area where steric hindrance may not be dominant is the nucleotide–aminopropanol–propionic acid chain f, which could be involved in reactions assisted by neighboring groups. One instance is the cleavage of vitamin B_{12} under highly acidic conditions (which suppress amide hydrolysis[37]) to give cobinamide and the 2′- and 3′-ribonucleotides.[1,3b] Other examples of neighboring group participation which result in fairly specific changes in this part of the molecule are at present being examined.

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THE SITE OF ABSORPTION OF VITAMIN B_{12} IN MAN

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FREELY diffusible substances such as glucose are rapidly absorbed from the upper part of the alimentary tract (Verzar and MacDougall 1936). It has been suggested that in man the absorption of vitamin B_{12} also takes place in the proximal small intestine (Castle 1953, Citrin et al. 1957). Clinical observations, however, conflict with this theory, for patients with lesions of the distal small bowel may be unable to absorb normal amounts of vitamin B_{12} and may develop megaloblastic anæmia (McIntyre et al. 1956, Booth and Mollin 1957a, Cooke et al. 1957, Gardner 1957). Furthermore, recent studies in animals have shown that under physiological conditions vitamin B_{12} is absorbed from the middle and distal thirds of the small intestine, but there is little or no absorption proximally (Booth, Chanarin, Anderson, and Mollin 1957, Booth and Mollin 1957a and b).

In this paper we report a study of the site of absorption of vitamin B_{12} in the human small intestine. The work is in three parts. Firstly, the distribution of radioactivity in the small intestine was measured in four patients at laparotomy 3 hours after the oral administration of test doses of radioactive B_{12}. Secondly, the absorption of vitamin B_{12} was studied in twelve patients who had undergone operations which involved resection or short-circuiting of small intestine. Finally, the incidence of vitamin-B_{12} deficiency in these twelve patients was assessed.

Materials and Methods

The radioactive vitamin B_{12} used in these studies was labelled with ^{56}Co or ^{58}Co (half-life 72 days).

Material of high specific activity (5 μC per μg.) was used for measuring the distribution of radioactivity in the small intestine at laparotomy. For measuring absorption by the fæcal-excretion technique in patients with intestinal lesions, the specific activity of the material used was 0·5–1·0 μC per μg.

Intrinsic factor was prepared by Lederle Laboratories from desiccated hog stomach, dissolved in water to a concentration of 1 mg. per ml., and stored at −20° C until required.

Plasma radioactivity.—20 ml. samples of venous blood were withdrawn and heparinised at timed intervals after giving the oral test doses of 1 μg. of radioactive B_{12} labelled with 5 μC of ^{56}Co. The radioactivity of 10 ml. samples of plasma was then counted in a modified annular-type scintillation counter (Haigh 1954). Background counting-rates on this counter ranged from 25 to 35 counts per minute.

The absorption of radioactive vitamin B_{12} was measured by the modified fæcal-excretion technique described by Booth and Mollin (1956). After the test dose was given, the fæces passed each day were collected in cylindrical tins, and the radioactivity was measured in a ring counter containing 32 Geiger-Müller tubes in parallel designed by Dr. J. E. Bradley. With this technique control subjects absorb from 0·26 to 0·87 μg. from an oral dose of 1 μg. of radioactive B_{12}, and patients with pernicious anæmia absorb up to 0·28 μg. from the same oral dose (Mollin, Booth, and Baker 1957).

Test doses of 1 μg. of radioactive B_{12} were given by mouth in 20 ml. of water after the subject had fasted for 12 hours. Nothing further was taken by mouth for at least 2 hours.

Serum-B_{12} levels were measured by the method described by Ross (1950). Using this method the levels in healthy subjects range from 140 to 900 μμg. per ml. Patients with pernicious anæmia have levels of less than 100 μμg. per ml. Some control subjects not suffering from pernicious anæmia may have levels between 100 and 140 μμg. per ml. (Mollin and Ross 1956).

Hæmatological methods.—Blood-counts were performed by the methods described by Dacie (1956).

Procedure

Observations at Laparotomy

Observations were made at laparotomy in two patients undergoing partial gastrectomy for peptic ulcer (cases 1 and 2), in one patient who was found to have an inoperable carcinoma of the stomach (case 3), and in one patient with pernicious anæmia in whom a suspected carcinoma of the stomach was not confirmed (case 4). Two patients (cases 1 and 2) were given the oral test dose alone, and the other two patients (cases 3 and 4) had the same oral dose together with 100 mg. of intrinsic factor. The absorption of the test dose was determined by the fæcal-excretion technique, and the changes in the plasma-radioactivity during the 24 hours following the oral dose were measured.

The distribution of radioactivity in the small intestine was measured at laparotomy 3 hours after the oral dose was given. This time was chosen because in man the absorption of vitamin B_{12} into the blood and tissues does not begin until about 3 hours after an oral dose (Booth and Mollin 1956).

An end-window Geiger-Müller counter (type GM 4, General Electric Co.) was used mounted in a lead sheath, and covered with a sterile mackintosh and towel. The counter was connected to a rate-meter, and the machine was used at 1200 V.

Background counting-rates were first recorded above the abdominal incision. These ranged from 1 to 3 counts per sec. Then, starting close to the duodenojejunal flexure, the radioactivity of the different segments was measured at successive positions, 12 to 18 in. apart down the length of the small intestine, by placing a loop of small intestine in close contact with the end of the counter. The last site was as near to the ileocæcal valve as possible. The whole procedure took 10 to 20 minutes in each patient.

Before and after use the counter was standardised by measuring the radioactivity of a 0·5 μC ^{60}Co standard placed directly against the window of the counter. This recorded 25 counts per sec. A ^{56}Co standard containing 0·5 μC was then counted; this recorded 12 counts per sec.

Observations after Operations on the Small Intestine

Radioactive B_{12}-absorption tests, using the fæcal-excretion technique, were carried out in twelve patients who had had operations involving resection or short-circuiting of the small intestine (cases 5 to 16). Each patient first had an oral dose of 1 μg. of radioactive B_{12} alone. If absorption was found to be subnormal, another oral dose was given with 50 mg. of intrinsic factor. Since malabsorption of vitamin B_{12} may be due to an abnormal bacterial flora in the small intestine rather than an intestinal lesion alone, a further test was carried out after a 4-day course of chlortetracycline (250 mg. 6-hourly) in the patients whose absorption of B_{12} was subnormal.

The serum-B_{12} levels and hæmatological findings were studied in all these patients, and the response to treatment with vitamin B_{12} was assessed in those in whom there was evidence of B_{12}-deficiency.

Results

Observations at Laparotomy

The absorption of radioactive B_{12} measured by the fæcal-excretion technique is given in table I, and the

TABLE I—ABSORPTION OF A TEST DOSE OF 1 μG. OF RADIOACTIVE VITAMIN B_{12} BY FOUR PATIENTS SUBJECTED TO LAPAROTOMY

Case no.	Amount of intrinsic factor given (mg.)	Amount of vitamin B_{12} absorbed (μg.)
1	0	0·50
2	0	0·35
3	100	0·52
4	100	0·71

corresponding changes in the plasma radioactivity are shown in fig. 1.

All four patients absorbed normal amounts of vitamin B_{12}, and the pattern of radioactivity in the plasma was similar to that found in controls not subjected to laparotomy (Booth and Mollin 1956). No radioactivity had appeared in the plasma of three of the four patients (cases 2–4) 3 hours after the oral dose, and only a small amount of radioactive material was found in the plasma at this time in the fourth (case 1). Peak radioactivity was at 8 or 12 hours in all four patients. These findings indicate that the operation did not interfere with absorption.

The distribution of the radioactive material in the small intestine of the four patients is shown in fig. 2. 3 hours after the oral dose, when little or no radioactive material had reached the blood, there was no radioactivity in the proximal small intestine. At this time all the radioactivity was in the distal half of the small intestine in three of the four patients (cases 1, 3, and 4), and in the distal two-thirds of the small intestine in the fourth (case 2). There was no significant difference in the results whether or not intrinsic factor was given with the oral test dose.

Absorption of Vitamin B_{12} after Small-intestinal Operations

The results of the absorption tests in the twelve patients who had undergone resection or short-circuiting of the small intestine, and the nature of their intestinal lesions, are given in table II.

The absorption of vitamin B_{12} was normal in the one patient (case 5) who had had 8 ft. of the jejunum resected, and in two patients (cases 6 and 7) in whom only 1 ft. of ileum had either been resected or short-circuited. The other nine patients, who had all had resection or short-circuiting of the ileum, absorbed subnormal amounts of vitamin B_{12}, and intrinsic factor did not improve their absorption.

There was no improvement in B_{12} absorption after a 4-day course of chlortetracycline in six patients in whom more than 6 ft. of ileum had either been resected or short-circuited (cases 10–12 and 14–16). Absorption became normal in one patient (case 8) who had had a right

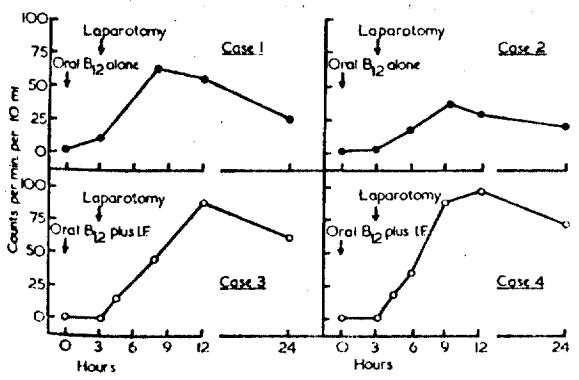

Fig. 1—Plasma-radioactivity in four patients given 1 μg. of ^{57}Co-labelled vitamin B_{12} by mouth, in whom laparotomy was performed.

hemicolectomy and resection of only 1 ft. of the ileum for regional ileitis, but who had developed a stricture at the site of the anastomosis.

Serum-vitamin-B_{12} Levels and Hæmatological Findings

The pretreatment serum-B_{12} levels and hæmatological data relating to the patients who had undergone resection or short-circuiting of the small intestine are also given in table II.

Normal serum-B_{12} levels and normal bone-marrows were found in five patients (cases 5–9). Three of them had absorbed vitamin B_{12} normally (cases 5–7), but in the two others (cases 8 and 9) the vitamin-B_{12} absorption was subnormal. Both these patients were seen 1 year or less after an operation on their distal intestine.

Subnormal serum-B_{12} levels (as low as in patients with pernicious anæmia) were found in seven patients who had had resection or short-circuiting of the distal small intes-

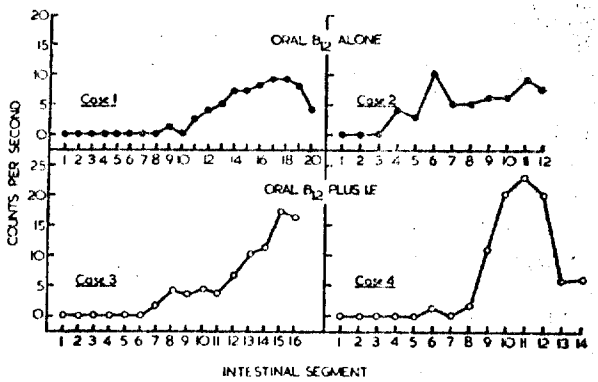

Fig. 2—Distribution of radioactivity in the segments of the small intestine of four patients subjected to laparotomy 3 hours after taking 1 μg. of ^{57}Co-labelled vitamin B_{12} by mouth. Segment 1 was close to the duodenojejunal flexure, and the last segment as near to the ileocæcal valve as possible.

tine (cases 10–16). Three of them (cases 14–16) had a megaloblastic anæmia. In four other patients (cases 10–13), seen 4 years or more postoperatively, there was little or no anæmia, but in the three whose bone-marrows were examined there were early or intermediate megaloblastic changes.

The response to parenteral injections of vitamin B_{12} (200 μg. monthly) was studied in four patients in whom there was evidence of B_{12}-deficiency (cases 10–12, and 16). In the one patient in whom there was a significant degree of anæmia (case 16) there was a reticulocyte response of 14·4% on the 6th day of treatment; the bone-marrow became normoblastic and the blood-count was normal 3 months later (hæmoglobin 13·1 g. per 100 ml.; red cell-count 4·9 million per c.mm.). In the other three patients (cases 10–12) macrocytosis disappeared from peripheral blood-films, and the bone-marrows became normoblastic.

Two other patients (cases 14 and 15) were given folic acid (10 mg. twice daily by mouth) together with parenteral vitamin B_{12}, and the hæmatological response was excellent in both.

Discussion

Observations at Laparotomy

The measurements made at laparotomy indicate that in man vitamin B_{12} is not absorbed in the proximal intestine. Our four patients all absorbed normal amounts of vitamin B_{12}, and the pattern of increase of radioactivity in the plasma was similar to that found in normal subjects

not subjected to laparotomy (Booth and Mollin 1956, Doscherholmen and Hagen 1957). 3 hours after the oral dose, when the plasma curves indicated that little or no absorption had taken place, most of the radioactive material was found in the distal small intestine and none was detected in the proximal segments (figs. 1 and 2). As vitamin B_{12} is not absorbed through the agency of intrinsic factor in the rectum or colon in man (Best et al. 1954, Citrin et al. 1957) we conclude that the radioactive B_{12} was absorbed from the ileum.

Effect of Small-intestinal Operations on B_{12} Absorption

The results of the B_{12}-absorption studies in the patients who had undergone resection or short-circuiting of the small intestine support the conclusion that in man vitamin B_{12} is absorbed in the distal small intestine. Resection of the jejunum does not interfere with B_{12} absorption (Cooke et al. 1957; our case 5), whereas absorption of vitamin B_{12}, with or without intrinsic factor, was invariably subnormal in the patients in whom more than 6–8 ft. of ileum had been resected or short-circuited (cases 9–16). This defect was not corrected by chlortetracycline (table II; see also case 4, table II of McIntyre et al. 1956), which may convert vitamin-B_{12} absorption to normal in patients who have jejunal diverticulosis or entero-enteroanastomoses (Badenoch et al. 1955, Halsted et al. 1956, McIntyre et al. 1956, Mollin, Booth, and Baker 1957). The malabsorption of B_{12} in the patients with no functioning ileum is therefore likely to be due directly to the anatomical defect rather than to the presence of abnormal bacteria in the distal small intestine. Malabsorption in these patients was not due to removal or short-circuiting of the ileocæcal valve, which did not interfere with B_{12} absorption (cases 6 and 7), except in the one patient who also had a stricture (case 8), and in whom absorption became normal after a course of chlortetracycline.

B_{12}-receptor Mechanism and the Site of Absorption

The reason why vitamin B_{12} is absorbed in the distal intestine is uncertain. Ineffective binding of B_{12} by intrinsic factor in the proximal intestine is apparently not the cause, because even when the test doses were previously mixed with intrinsic factor absorption still took place in the ileum (cases 3 and 4). An alternative explanation might be that the rapid motility of the proximal intestine hinders absorption in this area. But experimental evidence in the rat does not support this hypothesis, for B_{12} is taken up by the intestinal mucosa with great rapidity (Booth et al. 1957, Latner and Rainer 1957). Furthermore, the passage of barium through the small intestine was not unduly rapid in cases 10 and 16, and in case 10 other substances such as glucose, fat, and protein were absorbed normally.

The physiological absorption of vitamin B_{12} may therefore be a specific function of the distal small intestine in man, and possibly the ileum may contain a specific receptor mechanism for B_{12} absorption. The localised distribution of a specific receptor in the intestinal mucosa

TABLE II—ABSORPTION OF RADIOACTIVE VITAMIN B_{12}, SERUM-VITAMIN B_{12}-LEVELS, AND HÆMATOLOGICAL DATA IN TWELVE PATIENTS AFTER OPERATIONS ON THE SMALL INTESTINE

Case no.	Nature of intestinal lesion	Vitamin-B_{12} absorption (μg.)			Serum-vitamin-B_{12} level (μμg. per ml.)	Blood-count			Bone-marrow
		Test dose alone	Test dose + intrinsic factor			Hæmoglobin (g. per 100 ml.)	Red blood-cells (mill. per c.mm.)	Packed cell-volume (%)	
			Before chlortetracycline	After chlortetracycline					
5	Resection of 8 ft. of jejunum 5 mos. before ..	0·6	..	..	220	12·2	4·3	38	Normoblastic
6	Ileotransverse anastomosis 1 ft. proximal to ileocæcal valve 4 yr. before	0·5	..	..	360	14·5	4·7	47	Normoblastic
7	Resection of 1 ft. of terminal ileum. Ileocæcal anastomosis	0·46	..	..	500	14·8	5·1	46	..
8	Resection of 1 ft. of terminal ileum, and R. hemicolectomy 1 yr. before. Recurrence of regional enteritis caused stricture at ileocolic anastomosis	0·05	0·09	0·32	350	10·5	4·5	37	Normoblastic
9	Resection of all but proximal 4 ft. of small intestine, and R. hemicolectomy 6 mos. before, with end-to-end anastomosis	0·0	0·0	..	550	13·7	3·7	40	Normoblastic
10	Resection of terminal 2–3 ft. of ileum for regional ileitis 8 yr. before. Further resection of 3 ft. of ileum, with end-to-end anastomosis 6 yr. before	0·0	0·0	0·0	70	11·9	4·1	37	Early megaloblastic changes
11	Resection of terminal ileum, and R. hemicolectomy for regional ileitis 6 yr. before. Ileotransverse anastomosis 3 yr. before (6 ft. of ileum short-circuited)	0·19	0·13	0·0	100	13·7	5·5	40	Early megaloblastic changes
12	Extensive repair of intra-abdominal viscera after gunshot wounds 4 yr. before. Resection of all but proximal 4 ft. of small intestine, and R. hemicolectomy 2 yr. before	0·0	0·0	0·16	70	13·1	4·4	42	Intermediate megaloblastic changes
13	Resection of all but proximal 3 ft. of small intestine, and R. hemicolectomy 4 yr. before, with end-to-end anastomosis	0·0	..	..	85	14·6	4·8	44	..
14	Ileotransverse anastomosis 10 yr. before for regional enteritis. Further anastomosis 18 mos. ago. Ileum entirely short-circuited	0·0	0·0	0·0	25	6·7	3·1	26	Megaloblastic
15	Resection of uncertain amount of ileum, and R. hemicolectomy 4 yr. before. Further resection of 2 ft. of ileum 1 yr. ago. End-to-end anastomosis	..	0·14	0·0	<25	7·3	2·3	27	Megaloblastic
16	Resection of 4–6 ft. of terminal ileum, and R. hemicolectomy for carcinoma 4 yrs. before. Most of ileum short-circuited 2 mos. ago. Blind loop of terminal ileum	0·06	0·04	0·0	25	8·1	2·5	24	Megaloblastic

would explain the observation that the amount of B_{12} which can be absorbed through the intrinsic-factor mechanism is strictly limited (Glass et al. 1954). If there is such a receptor, specific defects of this absorption mechanism might occur. Theoretically, a congenital defect of a specific B_{12}-receptor could result in a clinical syndrome resembling pernicious anæmia, and B_{12} deficiency should be found regularly in patients who have disease of the ileum, or who have had operations which remove or short-circuit the ileum.

B_{12}-deficiency after Operations on the Small Intestine

The results in table II illustrate the frequency with which B_{12}-deficiency may follow operations on the ileum in man. Most of the patients described, who had no functioning ileum, had evidence of B_{12}-deficiency. The onset of signs and symptoms of this deficiency was insidious, and some years elapsed after operation before it became apparent. In cases 8 and 9 the serum-B_{12} levels were normal 6 and 12 months postoperatively, although neither patient was able to absorb vitamin B_{12} normally. This situation is analogous to that seen after total gastrectomy (Pitney and Beard 1955). In the remaining seven patients (cases 10–16), all of whom were seen at least 4 years after the operation on their ileum, the serum B_{12} had fallen to subnormal levels, and there was evidence of megaloblastic change in the six patients whose bone-marrows were examined.

These observations emphasise the importance of the ileum in the absorption of vitamin B_{12} in man. It is well known that patients who have had a total gastrectomy should either be treated prophylactically with vitamin B_{12} or be kept under regular hæmatological supervision. Our findings suggest that after operations involving resection or short-circuiting of the ileum, patients should be treated similarly.

Summary

Direct measurement of the distribution of radioactivity in the small intestine of four patients during the absorption of ^{56}Co-labelled vitamin B_{12} indicates that in man the site of vitamin-B_{12} absorption is the ileum. There may be a specific vitamin-B_{12}-receptor mechanism in this part of the small intestine.

In patients whose ileum had either been resected or short-circuited, the absorption of test doses of radioactive vitamin B_{12} was invariably subnormal and was unaffected by intrinsic factor or by previous treatment with chlortetracycline. Evidence of B_{12}-deficiency was found in many of these patients.

We therefore suggest that patients whose ileum has been resected or short-circuited should either be kept under close hæmatological supervision or be treated prophylactically with vitamin B_{12}.

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Evidence Concerning the Human Requirement for Vitamin B_{12}

Use of the Whole Body Counter for Determination of Absorption of Vitamin B_{12}

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The assessment of requirements of a nutrient can be approached from several standpoints: (1) observations of the occurrence or induction of deficiency in man; (2) response to therapy of clinical syndromes associated with deficiency; (3) metabolic studies; and (4) quantitative determination of intakes of the nutrient.

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This consideration of the current status of knowledge concerning vitamin B_{12} requirements employs several of these approaches, using data both from our studies and from others in the literature. Further, the application of the whole body counter as a safe, accurate and relatively simple means of appraising the absorption, distribution and excretion of tagged essential metabolites in man will be illustrated.

BACKGROUND

In reports[1,2] from this laboratory it has been argued that the development of vitamin B_{12} deficiency in patients with pernicious anemia, gastrectomized patients and Vegans is a comparable event and hence that patients with pernicious anemia may be used as examples of vitamin B_{12}-depleted subjects for studies of the usual human requirements. In a group of twenty patients, the minimal dose of parenterally administered vitamin B_{12} required to initiate maximal hemopoiesis was from 0.5 to 1.0 μg. daily.[2] Similar quantities of the vitamin maintained hematologic values at maximal levels. Considering the known efficiency of absorption by normal persons of such amounts of vitamin B_{12}, it was estimated that the minimal dietary needs for this factor can be met by an amount of 0.6 to 2.8 μg. of vitamin B_{12} from foods, and for most persons the range of 0.6 to 1.2 μg. should suffice.[2] Long-term observations on these patients[1] indicated that the demands for hemopoiesis equalled or exceeded those for other clinically recognizable physiologic functions and that macrocytosis

TABLE I

Analysis of Diets Patterned After Vanderbilt Cooperative Study of Nutrition in Pregnancy and NRC Standards

Diet	Vitamin B_{12} Content/Day (μg.)	Daily Calories	Protein (gm./day)
High cost	31.6 (4–85)	3,013	130 (98–155)
Low cost	16 (1.2–75)	2,439	116 (74–150)
Poor	2.7 (1.1–8.1)	1,117	(23–46)

NOTE: Assay, L. Leichmannii ATCC-7830.

TABLE II

Analysis of Pooled Rations, ICNND Surveys

Country of Origin	Vitamin B_{12} Content/Day (μg.)	% Males 20–45 Years old with Hemoglobin <12.0 gm.
Turkey	1.9	1.5
Peru	9.5	4.5
Libya	1.14–1.96 Mean—1.33	3.1
Korea	0.5	46.3
Ecuador	1.1–2.5 Mean—1.8	2.7

NOTE: Organism, L. Leichmannii ATCC-7830.

was the most sensitive indicator of vitamin B_{12} deficiency.

Recently published analyses of good and poor American diets provide pertinent data.[3] Table I summarizes the vitamin B_{12} content of poor, low cost and high cost diets as analyzed. (The assay procedure for vitamin B_{12} employed Lactobacillus leichmannii ATCC 7830.) The vitamin B_{12} content was as low as 1.1 μg. for the poor diets and as high as 85 μg. daily for high cost diets. The poor diets were patterned after the most restricted diets recorded by women studied in the Vanderbilt Cooperative Study of Maternal and Infant Nutrition. The diets with lowest content of vitamin B_{12} (average 2.7 μg.) were not associated with macrocytic anemia among the women. It is pertinent that in a survey of the vitamin B_{12} content of foods by Lichtenstein, Beloian and Murphy[4] of the U. S. Department of Agriculture, assays using L. leichmannii and Ochromonas malhemensis gave essentially equivalent results. Significantly, values obtained with O. malhemensis were higher in fifteen of twenty-seven foodstuffs analyzed, contrary to reported experiences for analysis of stool and urine. To quote from the summary of the Vanderbilt Study: "The dietary levels are equal to or greater than the estimates of adult requirements as based on the relatively meager evidence available for vitamin B_6, pantothenic acid, folic acid and vitamin B_{12}. They provide no support for a concept of widespread dietary lack of these factors. They afford an indication of the expected daily intake and, thereby, should offer some guide to a reasonable level of non-therapeutic supplementation for those who feel that such supplementation is desirable."[3]

A summary of the average vitamin B_{12} content in diets encountered in the course of studies made abroad by the Interdepartmental Committee on Nutrition for National Defense (ICNND) is provided by Table II. It is evident that anemia is prominent in Korea, where the vitamin B_{12} content of the diet averages but 0.5 μg. daily. The type of anemia was incompletely characterized although the mean corpuscular hemoglobin concentrations suggest that it was a hypochromic type of anemia. It is notable, however, that a level of 1 to 2 μg. daily does not result in any significant general incidence of anemia. Similar epidemiologic studies in areas in which iron deficiency is not limiting would be invaluable.

Important observations on absorption and excretion of vitamin B_{12} have issued from many laboratories, particularly pertinent are the suggestions of Reizenstein[5] and Gräsbeck[6,7] that data on vitamin B_{12} excretion in healthy subjects provide evidence that patients with pernicious anemia should receive from 4 to 10 μg. or more daily for the maintenance of body saturation with vitamin B_{12}.

We wish to report some recent observations on the absorption and biological half-life of vitamin B_{12} in normal subjects and in patients with vitamin B_{12} deficiency (pernicious anemia) and to interpret these in relation to vitamin requirements.

TABLE III
Subjects of the Study

Subject	Age (yr.) and Sex	Vitamin B_{12} Daily Dose μg./Frequency of Dose (wk.)	Diagnosis
L.K.	40,M	...	Diabetes mellitus
T.B.	27,M	...	Normal
W.M.	25,M	...	Normal
R.B.	41,M	...	Normal
J.T.	79,M	1.5/4	Pernicious anemia
M.M.	73,M	1.5/4	Pernicious anemia
J.C.	62,M	1.0/4	Pernicious anemia
O.T.	65,F	1.0/4	Pernicious anemia
P.H.	76,F	1.5/4	Pernicious anemia
H.C.	60,M	1.0/4	Pernicious anemia
S.W.	70,F	3.3/4	Pernicious anemia
P.D.	87,M	3.0/4	Pernicious anemia
A.D.	61,M	2.0/4	Pernicious anemia
A.P.	76,F	3.3/4	Pernicious anemia
B.V.	85,F	2.0/2	Pernicious anemia
M.F.	70,F	2.0/4	Pernicious anemia
B.A.	79,F	2.0/4	Pernicious anemia
F.L.	58,M	2.0/4	Pernicious anemia
C.H.	68,F	2.0/2	Pernicious anemia
J.J.	69,M	2.0/4	Pernicious anemia
R.J.	65,F	3.0/3	Azotemia, purpura simplex, normocytic anemia
S.N.	75,F	0.5/1	Pernicious anemia
R.R.	57, M	1.0/1	Pernicious anemia

METHODS

Data here reported are based on sixteen subjects for whom a long series of observations are available. An additional seven subjects, for whom only oral absorption studies are reported, are under study at present. At the time of the studies, all patients with pernicious anemia were in remission induced by the administration of regular doses of vitamin B_{12}. The total quantity of vitamin B_{12} given to each patient from the time of relapse on was known. Patients were given vitamin B_{12} at three dosage levels and are grouped as follows (Table III): (1) Those given weekly injections of vitamin B_{12} just sufficient to maintain hemopoiesis and normocytosis without providing a surplus for replenishment of body stores (3.5 and 7.0 μg. weekly or 0.5 to 1.0 μg. daily); (2) those given moderate doses (30 to 45 μg. monthly or 1.0 to 1.5 μg. daily); and (3) those given biweekly or monthly doses at higher levels (30 μg. per week to 60 to 100 μg. per month or 2.0 to 3.3 μg. daily).

Isotope counting was accomplished with a sodium iodide, thallium activated human whole body gamma ray scintillation spectrometer with the patient sitting beneath the crystal in a standard position.[8] Initial background counts of patients were made for thirty minutes. The radioactivity of solutions injected or capsules ingested was counted for a one minute period. Subsequent counts on the patients were for six minutes. Reproducibility of the six minute counts was ± 5 per cent with most of the variation constituting that associated with the randomness of the radioactive disintegration.

Each subject, while in a fasting state, was given approximately 0.5 μc. of Co^{60}-vitamin B_{12} (0.5 to 0.75 μc. per μg.). The residual whole body vitamin B_{12} radioactivity was determined on the next visit, which was usually two to four weeks later. If absorption was poor, the subject was given an injection of approximately 0.5 μc. (1 μc. per μg.) of the vitamin and radioactivity was determined on the next visit. The count at one month following injection was chosen as the standard for comparison with the value obtained following oral ingestion. Each patient served as his own standard for the calculation of percentage absorption by equation 1.

Equation 1:

$$\frac{R_0}{R_t} = I \times 100 = \% \text{ absorption}$$

R_0 = whole body c.p.m. remaining following elimination of unabsorbed vitamin B_{12}

R_t = whole body c.p.m. per μc. in patient one month following injection

I = μc. ingested orally.

The number of microcuries injected was determined by counting the contents in the syringe before and after injection and by comparison of these counts with those obtained from a known amount in the syringe. It was assumed that the distribution in the subjects after four weeks was similar whether the vitamin was received by injection or by absorption from the intestines. This procedure tends to increase the value for oral absorption in the patient with pernicious anemia by 5 to 10 per cent, for approximately this amount is lost in the first month following injection[11,25] which is only partially compensated for by the loss following oral absorption. For the normal subjects, who did not receive any injections, the 100 per cent value was taken as the activity measured immediately following ingestion of the capsule. The geometry of a 4 by 8 inch crystal, 0.48 M. from the patient, allows this as a first approximation. Our results compare with those of Reizenstein, Cronkite and Cohn[9] at Brookhaven National Laboratory.

For the half-life studies, the decay slope was de-

termined after repeated counts and extrapolated to zero time. The average half-life was obtained from a curve drawn through points for maximum fit determined by the method of least squares... The biological half life was calculated from equation II.

Equation II:

$$TB_{1/2} = \frac{TB_{1/2} \times TE_{1/2}}{TR_{1/2} - TE_{1/2}}$$

$TB_{1/2}$ = biological half-life
$TR_{1/2}$ = intrinsic half-life of Co^{60} (1,923 days)
$TE_{1/2}$ = effective half-life (from curve).

The slope of biological decay or decay constant (K) was determined by use of equation III.

Equation III:

$$K = \frac{0.693}{TB_{1/2}}$$

K = slope of biological decay (decay constant)
$TB_{1/2}$ = biological half-life.

RESULTS

Absorption Studies

The results of absorption studies are summarized in Table IV. There is a wide separation between the healthy subjects and the patients with pernicious anemia. The range of absorption in normal subjects is 45 to 80 per cent with a mean of 70 per cent; for the patients it ranged from 0 to 17 per cent with a mean of 3.3 per cent.

The values for per cent absorption by normal subjects and patients are similar to those of Reizenstein and colleagues[9] who reported 38 to 80 per cent and 0 to 8.8 per cent, respectively.

TABLE IV
Per Cent Oral Absorption of Co^{60}-Vitamin B_{12}

Subjects	No. Subjects	0.5 μg./dose 0.5 μc. Co^{60} ± Vitamin B_{12}	S.E.* Mean
Nonpernicious anemia	5	45–80% Mean—70%	± 6.58
Pernicious anemia	18	0–17% Mean—3.25%	± 0.87

* Standard error.

They are also similar to values obtained previously from studies utilizing measurement of urinary radioactivity, stool analysis or measurement of radioactivity over the liver. The present technic appears to be superior to other methods commonly used by reason of its simplicity and accuracy and the possibility of employing minimal dosage of radioactivity for long-term studies. Stool collections are unnecessary and the added uncertainty of variations in renal excretion is avoided. Further, large flushing doses of vitamin B_{12} are not required and the attendant obscuration by this flushing of subsequent study is avoided. The technic employing the whole body counter has been successfully applied at the radioisotope center at Vanderbilt University[8] to the measurement of absorption, distribution and excretion of numerous elements, including iron, sodium, potassium, cesium *inter alia*.

Biological Half-Life

Sixteen subjects have been observed from eight months to over two years after receiving the labeled vitamin. The calculated biological half-life for each is listed in Table V. Representative observations are depicted in Figures 1 through 5.

The differences in biological half-life between normal subjects and patients with pernicious anemia are probably not significant. The highest turnover rates were observed in two subjects, one with diabetes mellitus and one with azotemia and purpura. These are unexplained. There is no correlation between the frequency or magnitude of the dose of vitamin B_{12} in patients with pernicious anemia and the rate of loss of labeled vitamin from the body. This observation is further supported by the fact that there was no statistically significant change in rate of loss of vitamin B_{12} after therapy was discontinued in six patients who had been receiving vitamin B_{12} parenterally (Table V).

COMMENTS

It is necessary to identify the assumptions in the interpretations made. It is assumed that labeled vitamin B_{12} is distributed in a manner similar to nonlabeled vitamin B_{12} in the body

TABLE V
Biological Half-Life of Vitamin B_{12} in Man

Subjects	μg. of Vitamin B_{12} Given	During Vitamin B_{12} Therapy				After Vitamin B_{12} Therapy			
		Length of Period	No. of Observations	% Loss per Day	Biological Half-Life (days)	Length of Period	No. of Observations	% Loss per Day	Biological Half-Life (days)
Normal Subjects									
R.B.	Dietary	478	9	0.12	577	...	...	...	...
T.B.	Dietary	430	6	0.15	462	...	...	...	...
W.M.	Dietary	430	4	0.09	770	...	...	...	...
Patients without Pernicious Anemia									
R.J.*	Dietary + 65/3 wk.	173	7	0.23	301	398	10	0.21	330
L.K.†	Dietary	487	7	0.21	330	...	...	...	...
Patients with Pernicious Anemia									
S.N.	3/wk.	668	61	0.15	462	...	...	...	...
R.R.	7/wk.	354	20	0.15	458	384	15	0.14	495
H.C.	30/4 wk.	420	3	0.11	630	...	...	...	...
J.C.	30/4 wk.	344	12	0.09	770	405	12	0.10	693
J.T.	45/4 wk.	152	5	0.14	495	337	7	0.11	630
M.M.	45/4 wk.	220	4	0.16	433	348	6	0.11	630
M.F.	60/4 wk.	240	5	0.17	408	...	...	...	...
J.J.	60/4 wk.	364	9	0.14	495	...	...	...	...
F.L.	60/4 wk.	420	3	0.11	630	...	...	...	...
C.H.	30/2 wk.	420	8	0.13	530	...	...	...	...
B.V.	60/2 wk.	220	6	0.12	577	345	15	0.12	577

* Azotemia plus purpura.
† Diabetes mellitus.

after a period of approximately sixty to ninety days and that the labeled cobalt in the tissue is in the form of vitamin B_{12}. It is further assumed that the "pools" or "compartments" of vitamin B_{12} attain equilibrium and that losses from any compartment are reflected throughout after this period. There is evidence to support each of these assumptions.

During the first several weeks following absorption or injection of Co^{60}-vitamin B_{12}, there are wide fluctuations of counts which probably correspond to redistribution of the vitamin through various compartments. Usually after a month, and invariably after two months in the case of injected vitamin B_{12}, the decay rate of radioactivity in the body becomes relatively constant. Normal subjects who did not receive an injection maintained a steady rate of decline of radioactivity following the stabilization period which corresponds to a biological half-life of 462 to 770 days and a decay constant of 0.09 to 0.15 per cent of residual counts daily (Fig. 1 and 2, Table v). Similarly, patients with pernicious anemia displayed decay constants which ranged from 0.11 to 0.17 per cent per day (Fig. 3, 4 and 5, Table v).

As an approximation, this would imply that the daily loss of vitamin B_{12} in both normal subjects and patients with pernicious anemia approaches a first order function with the absolute amount lost being a function of the size of the pool. Table VI gives data from Ross

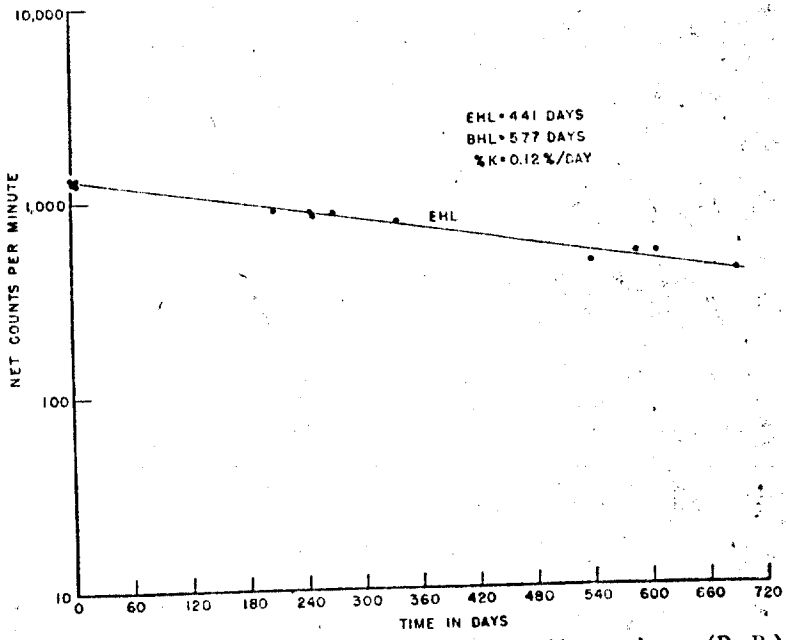

FIG. 1. Loss of Co^{60}-vitamin B_{12} in forty-one year old normal man (R. B.). (Oral tracer dose—0.53 μc. Co^{60}-vitamin B_{12} per 0.75 μg.)

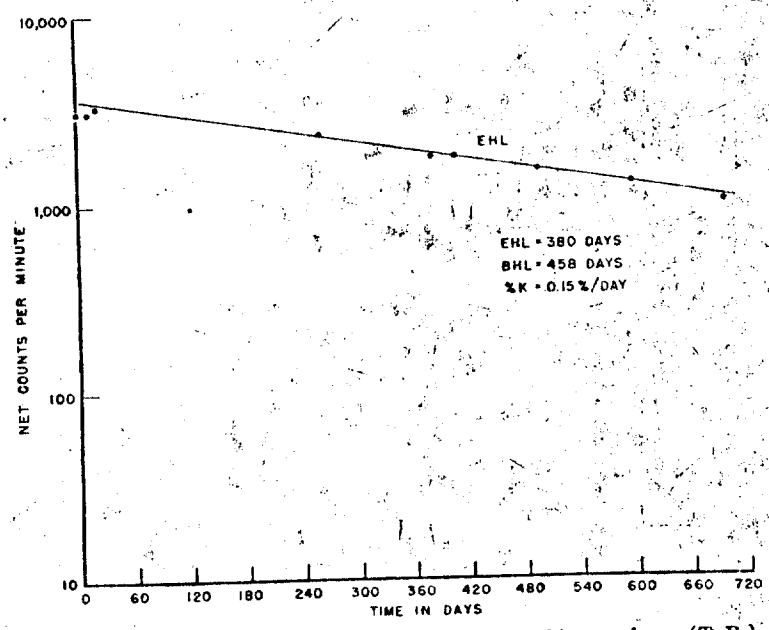

FIG. 2. Loss of Co^{60}-vitamin B_{12} in twenty-seven year old normal man (T. B.). (Oral tracer dose—0.53 μc. Co^{60}-vitamin B_{12} per 0.75 μg.)

and Mollin obtained on autopsy material[13] and from Wolff,[14] Swendseid[15] and their associates on the vitamin B_{12} content of the human liver in healthy subjects and in patients with pernicious anemia in relapse. Table VII is a compilation of data on the vitamin B_{12} content of the liver in control subjects and in patients with pernicious anemia in remission.

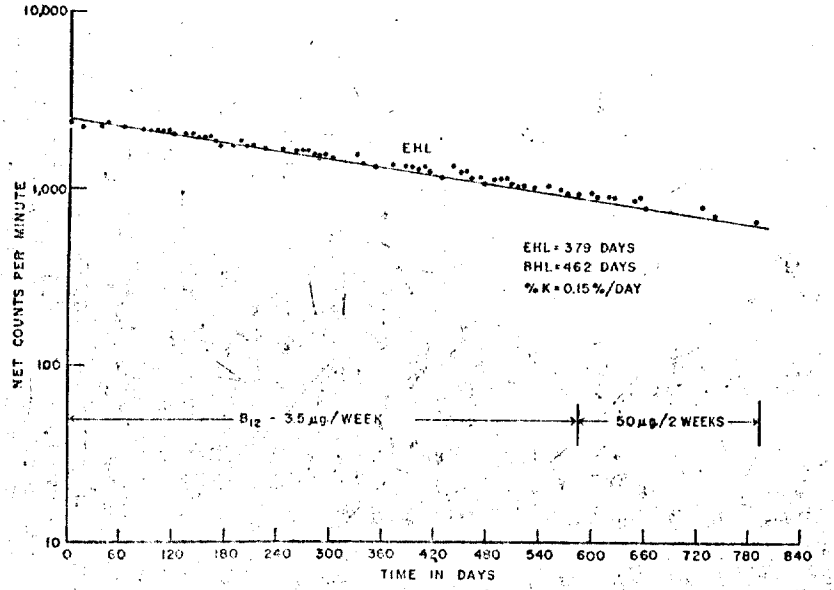

FIG. 3. Loss of Co^{60}-vitamin B_{12} in seventy-five year old woman with pernicious anemia (S. N.) (Subcutaneous tracer dose of 0.53 μc. Co^{60}-vitamin B_{12} per 0.53 μg.)

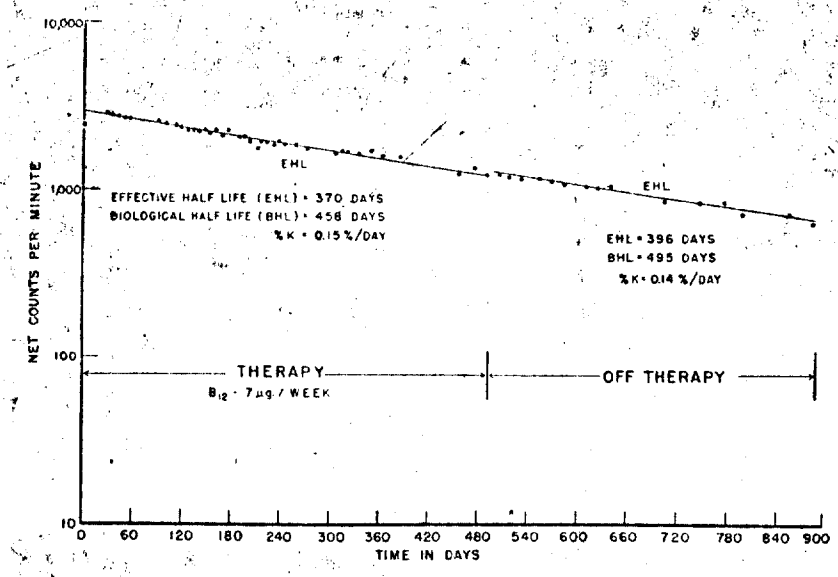

FIG. 4. Loss of Co^{60}-vitamin B_{12} in fifty-seven year old man with pernicious anemia (R. R.). (Subcutaneous tracer dose of 0.53 μc. Co^{60}-vitamin B_{12} per 0.53 μg.)

It is evident that the stores of vitamin B_{12} in both treated patients and in patients in relapse are well below those of normal subjects. Table VIII shows the comparison of turnover rates in three normal subjects and in two patients treated with minimal doses of vitamin B_{12}. One patient (R. R.) was in hematologic and neurologic relapse, and remission occurred on a regimen of 7 μg. of vitamin B_{12} per week. His serum vitamin B_{12} levels have been consistently below 100 μμg. per ml. In the other patient (S. N.) relapse occurred while on a regimen of 3.5 μg. per week. Values for decay constants in such close agreement for persons

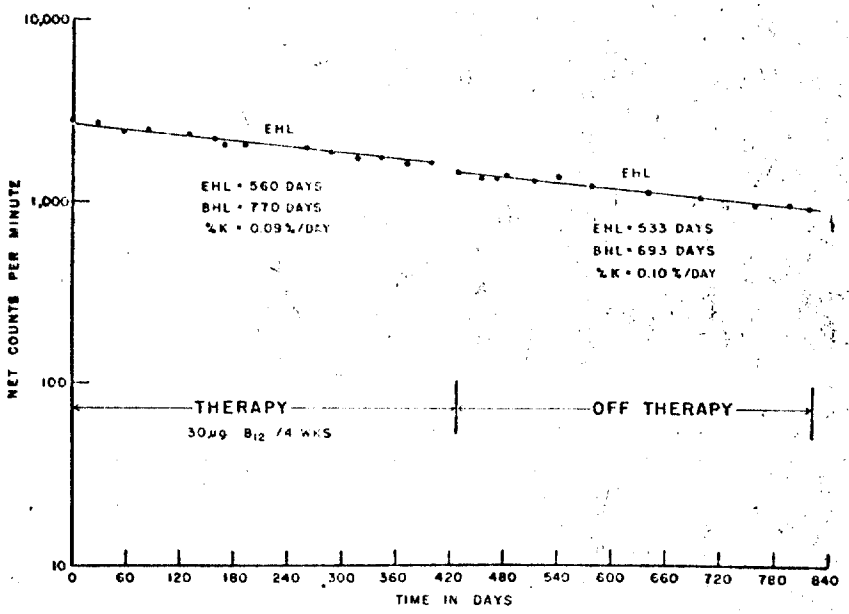

FIG. 5. Loss of Co^{60}-vitamin B_{12} in sixty-two year old man with pernicious anemia (J. C.). (Subcutaneous tracer dose of 0.51 μc. Co^{60}-vitamin B_{12} per 0.51 μg.)

TABLE VI
Vitamin B_{12} Content of Human Liver

Investigator	Normal Subjects	Subjects with Pernicious Anemia Relapse
Swendseid, Hvolboll, Schick and Halsted[15]	0.75 to 1 μg./gm.	
Ross and Mollin[13]	1,500 μg./total liver	0.1 μg./gm. to as low as 10 μg./total liver
Wolff, Drouet, Karlin and Rauber[14]	0.8 μg./gm.	
Pitney and Onesti[18]	0.78 μg./gm.	

TABLE VII
Vitamin B_{12} Concentration in Liver of Normal Control Subjects and Patients with Pernicious Anemia in Remission

Material Studied	Normal Subjects		Patients with Pernicious Anemia in Remission	
	No.	Concentration	No.	Concentration
Liver biopsy[16]	12	1.9 μg./gm. liver	3	1.3 μg./gm. liver
Autopsy[13]	13	1.5 mg./total liver	5	0.7 mg./total liver
Liver biopsy[14]	6	0.82 gm./gm. liver	5	0.2 μg./gm. liver
Liver biopsy[18]	10	0.78 μg./gm. liver		

with body stores at such widely differing levels support the view that daily loss approaches a first order function. An expansion of the pool of the patient with pernicious anemia by vigorous therapy theoretically would then result in the loss of increased amounts of vitamin B_{12} via stool, urine and possibly epithelial desquamation.

TABLE VIII

Comparison of Patients with Pernicious Anemia and Markedly Depleted Body Stores of Vitamin B_{12} to Normal Subjects

Subject	Length of Study Period (days)	No. of Observations	Biological Half-Life (days)	% Loss/Day
R.R.*	738	35	495	0.14
S.N.*	668	61	462	0.15
R.B.	478	9	577	0.12
T.B.	430	9	462	0.15
W.M.	430	4	770	0.09

* Patients with pernicious anemia.

This concept of excretion as a first order function has been expressed by Gräsbeck[6,7] and hinted at by Reizenstein[5] and Adams.[17] Our data, which are comprised of a longer series of observations, extend this concept and we interpret it and its ultimate significance differently.

Earlier it was stated that certain assumptions are inherent to the interpretation of data on biological half-life; namely, the identity with vitamin B_{12} of the residual radioactivity in tissues, the similarity of distribution of injected and body vitamin content and reflection by all body compartments of losses from any compartment. The following evidence supports these considerations. Schloesser, Desphande and Schilling,[19] Glass and Mersheimer[20] and Gräsbeck et al.[21] have established in man and animals that the radioactive, stored component corresponds to vitamin B_{12} by chromatographic and microbiologic correlation of vitamin B_{12} activity and radioactivity. The latter group has shown that the specific activity within various organs in the rat is essentially the same after an equilibration period of one month. Data presented in Tables VI and VII and by Blum and Heinrich[22] indicate that the relative vitamin B_{12} content in various organs of normal subjects and of patients with pernicious anemia, with or without treatment, remains proportional. Baker and associates[23] have published data indicating that the vitamin B_{12} level of erythrocytes and plasma of patients with pernicious anemia is reduced in value to an extent paralleling the other organs. These findings support the thesis that the various pools are in equilibrium and that the stored radioactivity corresponds to vitamin B_{12}.

In an abstract, Reizenstein[24] reports on studies of the half-life of Co^{60}-vitamin B_{12} utilizing the whole body counter. He found that there was a progressively decreasing rate of excretion of radioactivity with time and that even at 250 days the rate continued to decrease. Details of this study are not yet available, but we have followed our normal subjects for over a year and have not observed any evidence of a decrease in the slope of decay after a period, at most, of several months. Loss of radioactivity in each of the three normal subjects has maintained a constancy which has extended to as long as 479 days to date. This constancy warrants the assumption that general equilibration is complete in a quantitative sense even though final equilibration may be incomplete for certain tissue components which have exceedingly low turnover rates. It is recognized that each tissue has a spectrum with respect to constituent turnover time and it is not unreasonable to consider that certain constituents involved with vitamin B_{12} do have an even slower turnover rate than the period here involved. The half-life figures probably represent an average figure covering a wide spectrum of half-life values for various tissue components.

The implications of the concept presented are as follows: The body is capable of storing significant amounts of vitamin B_{12} (790 to 11,900 μg. in the normal subject).[13,25] The size of the body store is the major determinant of the quantity of vitamin B_{12} lost daily. Other contributing factors, such as rate of degradation, turnover and inactivation of the active form of vitamin are of a magnitude which probably is minor when compared with the contribution of the pool size. The larger the body stores, the larger the excretion. This relationship of body stores to excretion is even more important in patients with pernicious anemia who may well have a limited reabsorption capacity for vitamin B_{12} which enters the intestine in the bile.[11,25] The body maintains a balance at various levels of intake and storage. Low levels of intake and small pool size are associated with low levels of excretion. It

does not follow that there is attached to any particular level of storage or excretion an especial advantage with respect to optimal health. Grasbeck and Reizenstein have argued that the conventional treatment for pernicious anemia is inadequate; that maintenance therapy should be such as to saturate the body stores to the levels of healthy normal subjects. The amounts they suggest approximate 4 to 10 μg. per day.[5-7] They advance the suggestion that neurologic complications, decreased fertility, degenerative states and possibly the development of gastric neoplasm, gallbladder disease and other usual complications could be alleviated or possibly averted by the use of saturating doses of vitamin B_{12}. There is no acceptable evidence for these suggestions. There is no evidence at present that the administration of large doses of vitamin B_{12} prevents any of the corollaries of pernicious anemia such as gastric atrophy, gastric polyps or achlorhydria. The toll of subacute combined degeneration appears to be a function of the completeness of depletion and length of time the deficiency existed, rather than being related to the treatment dose. Indeed, less vitamin B_{12} seems to be required to prevent combined system disease than macrocytosis in pernicious anemia.[2]

As previously stated, one of the patients in this series (R. R.), a fifty-five-year old Negro man, has been treated with a weekly dose of 7 μg. of vitamin B_{12} without preliminary replenishment of body stores; he experienced complete remission of the hematologic picture in a matter of weeks and of the neurologic defects over a period of six months. This occurred in the face of body stores which remained "depleted" as measured by serum levels* of 50 and 90 $\mu\mu$g. per ml., two and six months after the start of treatment. The experience at the Vanderbilt Hospital and other clinics over the years does not provide evidence to suggest that failure to saturate body stores with vitamin B_{12} (by present standards) is detrimental to patients. The protracted well-being of the patient, the maintenance of functional good health without evidence of glossitis, intestinal symptoms, neurologic deficit or macrocytosis cannot be disregarded. On the basis of this and previous data, one might predict that it would be difficult to attain and sustain full body stores at saturation of vitamin B_{12} by injection.

The estimates at which we have arrived with regard to turnover of vitamin B_{12} are compatible with clinical observations concerning the length of time required for the signs and symptoms of a lack of vitamin B_{12} to develop in patients who either have undergone total gastrectomy or from whom vitamin B_{12} therapy has been withdrawn.[1] Ordinarily relapses occur in six months to six years, an observation made repeatedly. This period of time for exhaustion of body stores is predicted from our data. Relapse would be expected to occur when the body stores of vitamin B_{12} are approximately 500 μg. or less.[6] If initial body stores are 4,000 μg., then this level would be expected in about five years and if they are 10,000 μg. at about eight to ten years. A slower turnover rate, as suggested by Reizenstein,[24] does not coincide with the clinical observations and, in fact, would have abnormalities occurring at fourteen to twenty years under similar circumstances.

An additional factor for consideration suggested by the rate of loss of isotope from the body (a body store of 7.00 to 11,300 μg. would imply a loss of 0.79 to 11.9 μg. daily at the decay rate of 0.1 per cent) is the limits of absorption of vitamin B_{12} by the normal subject. Previous studies in normal adults, or in patients with pernicious anemia given supplements of intrinsic factor, indicated that absorption is approximately proportional to the log dose of vitamin B_{12} with an upper limit of approximately 2 μg. for a 100 μg. dose.[26] Glass, Boyd and Stephanson[27] have postulated a mucosal block, analogous to that with iron, which prevents the absorption of more than 1.5 μg. from a 50 μg. dose. Swendseid et al.[28] set an upper limit of 1.4 μg. These studies, in common, used crystalline vitamin B_{12} as the test material. Vitamin B_{12} occurs in food in other than the cyano or crystalline form.[29] Cyanide treatment of bound vitamin B_{12} displaces

* Courtesy of Dr. G. Goldsmith, Department of Medicine, Tulane University School of Medicine, New Orleans, Louisiana. Assay organism, L. leichmannii.

it from its binding, yet it is the bound form in liver which is absorbed at a level of 30 per cent of a 22 μg. dose.[30] The effect of food and digestion upon the secretion of intrinsic factor or any other absorption-stimulating factor which may be present in gastric or intestinal secretions, should be considered. This is illustrated by the increased absorption of crystalline vitamin B_{12} following stimulation of gastric secretion by carbamylcholine.[31] Abels and associates[32] have reported absorption of vitamin B_{12} as high as 35 per cent of a 10 μg. dose in a normal person by the concomitant administration of human gastric juice. This is to be compared with much poorer absorption with the use of heterologous forms of intrinsic factor.[33] Studies need to be made in man to determine (under varying conditions) the limits of absorption of labeled vitamin B_{12} present in foodstuffs in its natural state.

CONCLUSIONS

The considerations presented provide indications as to a "floor" on vitamin B_{12} needs. Minimal requirements will maintain health and normal hemopoiesis in most subjects who have a small body pool and who are operating at an efficient physiologic economy. These are at the level of 0.6 to 1.2 μg. daily. Storage bins filled to the "ceiling," if achieved, would be associated with a considerable loss of physiologic economy. A larger body pool results in a larger loss; to maintain maximum body stores would require high levels of intake, especially when injected as crystalline cobalamine. One may argue the philosophical question whether the patient benefits from a full body store of the nutrient, but it is evident that there is a wide range of healthy adaptability for vitamin B_{12} (as with most nutrients). These limits, concerning which we believe one may be reasonably definite, represent variations in rate of use, loss and excretion (principally the latter) of the injected, crystalline vitamin, when added to body pools of various sizes.

Knowledge concerning the normal absorption from the gastrointestinal tract of different forms of the vitamin in the natural state, the influence of various foods upon absorption, the mechanisms for and limits of normal gastrointestinal absorption and the distribution in foodstuffs and diets must be clarified before the *dietary requirements* of vitamin B_{12} can be stated precisely and with assurance.

SUMMARY

An example of the use of the whole body counter as a safe, simple and accurate means of assessment of absorption, distribution and excretion of a metabolite, vitamin B_{12}, is presented.

Results of absorption studies in five subjects without pernicious anemia and eighteen patients with pernicious anemia are presented. The range of absorption in the subjects without pernicious anemia is 45 to 80 per cent with a mean of 70 per cent and in those with pernicious anemia 0 to 17 per cent with a mean of 3.25 per cent.

Studies of biological half-life of vitamin B_{12} are presented in sixteen subjects (eleven with pernicious anemia and five without), including three normal subjects, who have been observed for as long as two years. There was no significant difference in decay constants between those with pernicious anemia and the normal subjects. The values ranged from 0.09 to 0.15 per cent loss per day of labeled vitamin in normal subjects, and from 0.11 to 0.17 per cent loss per day in eleven patients with pernicious anemia. These figures correspond to a biological half-life of 407 to 770 days.

The proposal is made and support cited for the concept that the daily need for injectable vitamin B_{12} in patients with pernicious anemia reflects almost entirely the loss of the vitamin from the body. Despite the marked difference in the size of the body stores of vitamin B_{12} in normal subjects and in those with pernicious anemia, daily loss rate constants are essentially identical.

Parameters are identified which require study before dietary requirements levels can be set more precisely.

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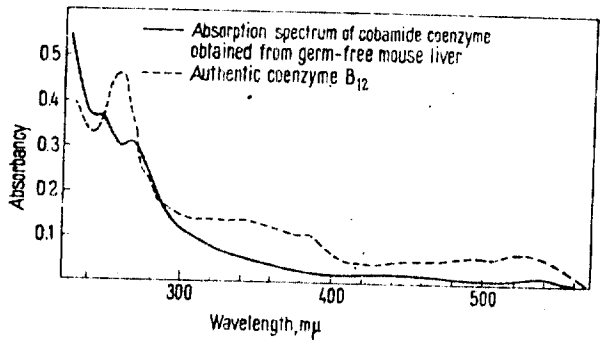
Isolation of Coenzyme B_{12} from Liver of Germ-free Mice

Recent reports from BARKER's laboratory[1,2] have summarized studies leading to the discovery and characterization of several forms of cobamide coenzymes which are light-sensitive derivatives of vitamin B_{12} containing an adenosyl moiety attached to the cobalt atom in the corrin ring. The obligatory participation of cobamide coenzymes has been demonstrated in the enzyme-catalyzed isomerization of glutamate to β-methylaspartate[3], the conversion of methylmalonyl-coenzyme A to succinyl-coenzyme A[4-6], and the dismutation of 1,2-diols to the corresponding deoxyaldehyde[7]. The synthesis of B_{12} coenzymes by enzyme preparations obtained from several bacterial species has been amply demonstrated[8-11], and the presence of cobamide coenzymes in human, chicken, lamb and rabbit liver has been reported[12]. Recently the conversion of Co^{60}-labeled cyanocobalamin to its coenzyme form has been observed in the rabbit *in vivo*[13]; however, there has been no direct demonstration of the enzymatic synthesis of B_{12} coenzymes in preparations obtained from mammalian tissue *per se*. While the existence of such synthesis seemed likely, the possibility still remained that the formation of these materials might occur exclusively through the activity of intestinal flora, in view of the facile synthesis of cobamide coenzymes catalyzed by extracts of certain microorganisms. It seemed reasonable, therefore, to examine the livers of animals raised under germ-free conditions for the presence of B_{12} coenzymes.

The germ-free animals studied were drawn from a colony of white Swiss mice which has been maintained in steel isolators in the LGAR for approximately 4½ years. The colony was obtained originally from the Lobund Institute, University of Notre Dame. The animals are fed a semi-synthetic diet[14] which is sterilized by steam at 255°F for 25 min. They are checked approximately every 2–3 weeks for bacteria, fungi, and parasites according to techniques described previously[15]. The control mice used for comparison were drawn from a colony which is maintained in an ordinary animal room, and which harbors a varied flora. This colony was derived from the germ-free colony. Breeders from the latter are removed periodically from the isolators and added to the control colony in an effort to keep the gene pools similar. These animals are fed the same sterilized diet given the germ-free.



Absorption spectrum of cobamide coenzyme isolated from germ-free mouse liver and authentic coenzyme B_{12}. The solution was adjusted to a final concentration of 0.01 *M* potassium phosphate buffer at pH 6.7. An exact comparison of the spectrum of the cobamide coenzyme obtained from the germ-free mouse liver tissue and crystalline coenzyme B_{12} cannot be made at this stage of purification[12]. The spectum shown here compares favorably with those reported by TOOHEY and BARKER[12] for the coenzymes obtained from lamb, human and rabbit liver.

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Several adult mice of both sexes from each colony were sacrificed and cobamide coenzymes were isolated from the pooled livers according to TOOHEY and BARKER[18]. Essentially the method consists of the preparation of an acetone powder followed by the extraction of cobamide coenzymes into hot 80% ethanol. The ethanol was removed and after extraction of phospholipids with ether, the solutions were passed through an anion exchange column, the cobamide coenzymes were concentrated with phenol, passed over a cation exchange resin at neutral pH and finally adsorbed and eluted from a 5 × 18 mm column of Dowex-50-Na^+H^+. The absorption spectrum of the purified coenzyme is shown in the Figure along with the spectrum of authentic coenzyme B_{12}. The amount of cobamide coenzyme in the various fractions was determined by the glutamate isomerase assay[18] (Table).

Determination of cobamide coenzyme in mouse liver. This assay was made after passing the coenzyme-containing solution through Dowex-50 at pH 7.0

Condition of mice	Control	Germ-free
Weight of pooled liver tissue in g	10	26
Cobamide coenzyme activity*, mμmoles/kg	190	239

* The values are expressed in terms of the activity relative to that of crystalline coenzyme B_{12} in the glutamate isomerase assay system.

The results indicate that the livers of germ-free mice contain significant quantities of cobamide coenzyme and the values obtained compare very well with those obtained with mice harboring a flora. They also approximated values reported for human and lamb liver, which were 220 and 180 mμmoles, respectively, per kg of fresh liver at the same purification step. It was not possible to identify the specific base of the coenzyme obtained from the germ-free mice due to the limited quantity of tissue available.

The available information on the metabolic activities in tissues from germ-free animals has not indicated any profound abnormalities. Thus, one might have concluded that B_{12} coenzymes would be found in germ-free mouse liver if mammalian methylmalonyl-coenzyme A isomerase catalyzes an obligatory, or even a quantitatively significant, reaction for normal metabolic function.

Zusammenfassung. Die enzymatische Synthese von Cobamid-Coenzymen ist aus Extrakten, die von Mikroorganismen gewonnen wurden, gut bekannt, nicht aber von Säugetiergeweben. Unsere Befunde mit Cobamid-Coenzymen in der Leber von keimfreien Mäusen scheint anzuzeigen, dass die Verwandlung von Vitamin B_{12} in Coenzymform von Enzymen der Säugetiergewebe katalysiert werden kann.

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Laboratory of Neurochemistry, National Institute of Neurological Diseases and Blindness, and Laboratory of Germ-free Animal Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda (Maryland, U.S.A.), March 25, 1963.

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S. Afr. J. med. Sci. (1961), 26, 1-3.

SERUM VITAMIN B_{12} LEVELS IN SOUTH AFRICAN WHITE AND BANTU SUBJECTS

by

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It would not be surprising if serum vitamin B_{12} levels in the South African Bantu varied widely. The intake of animal protein, a major dietary source of vitamin B_{12}, is often low in the Bantu [Ockerse *et al.*, 1953], and this might result in low serum vitamin B_{12} levels. Liver disease, common in the Bantu, may be associated with high serum vitamin B_{12} levels [Jones *et al.*, 1957]. Results of serum vitamin B_{12} assays in healthy Bantu subjects have been published by Adams and Forbes [1957]. Using Euglena gracilis as test organism, these authors found serum vitamin B_{12} to range from 481-1701 (mean 1020) $\mu\mu$g./ml. in 16 healthy Bantu subjects. In 16 Europeans serum vitamin B_{12} ranged from 348-843 (mean 534) $\mu\mu$g./ml. The markedly higher mean level in the Bantu was statistically significant.

This paper presents further results of serum vitamin B_{12} estimations in White and Bantu subjects.

MATERIAL

Equal groups of 100 Bantu and 100 White subjects were studied. All were blood donors, who had been screened to exclude anaemia before being bled. Approximately 10 ml. blood was drawn by venipuncture, the serum separated, and stored at —20°C until assayed.

METHOD

The method used was modified from that described by Spray [1955].

Culture medium

The culture medium is Vitamin B_{12} Assay Medium (Difco), prepared according to the formula of Capps *et al.* [1949]. After rehydration, the pH of the medium is adjusted to 5·5. The addition of phenobarbitone, recommended by Spray, was not found to enhance growth of the test organism, and was not used.

Stock cultures and preparation of inoculum

The test organism is Lactobacillus leichmanii ("Glaxo strain"). Stock cultures are maintained on Microassay Culture Agar (Difco) by sub-culturing at weekly intervals. On the evening before an assay, a loopful of organisms from the stock culture is transferred to a bottle of Microinoculum Broth (Difco) and incubated overnight and the following day at 37°C. The culture is centrifuged, the supernatant decanted, and the organisms suspended in 10 ml. of single strength medium. The centrifuging is repeated and the organisms finally resuspended in 10 ml. single strength medium. This suspension is used as the inoculum.

Preparation of serum extracts

In order to liberate the protein-bound vitamin B_{12}, sera are heated in the presence of acetate buffer and cyanide. Serum (1·5 ml.) is added to 0·75 ml. of sodium acetate buffer and 0·3 ml. of 0·1% sodium cyanide. The mixture is made up to 15 ml. with water resulting in a 1:10 dilution of serum, and then placed in a boiling water bath for 30 minutes. After cooling the bottle is shaken, the precipitated protein is filtered off, and the clear supernatant used for assay.

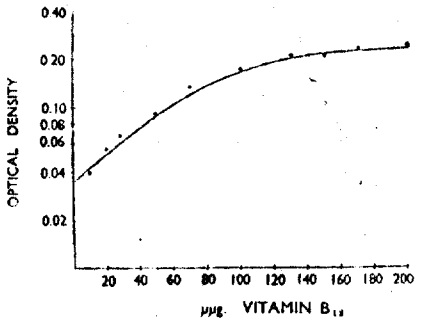

Fig. 1

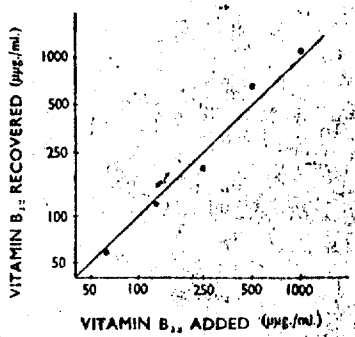

Fig. 2

Fig. 1. Standard curve plotted on 2 cycle semi-log. paper.
Fig. 2. Recoveries of vitamin B_{12} added to serum.

Standard Vitamin B_{12} solutions

For the construction of a standard curve, dilutions of a 50 μg./ml. solution of vitamin B_{12} "cytamen" (Glaxo) are made. A convenient range was found to be between 1 and 200 $\mu\mu$g./tube, and standards equivalent to 1, 10, 20, 30, 50, 70, 100, 120, 150, 170 and 200 $\mu\mu$g./tube are prepared.

Assay method

One ml. of the serum extract is diluted to 5 ml. with water and to this, and to 5 ml. aliquots of the standard vitamin B_{12} solutions, are added 5 ml. of double strength medium. All specimens and standards are set up in triplicate. After autoclaving and cooling the tubes are inoculated, and then incubated at 37°C for about 40 hours. After incubation the tubes are shaken, and the turbidity read against an uninoculated blank (5 ml. distilled water + 5 ml. double strength medium) on a Unicam photo-electric colorimeter. The standard curve (Fig. 1) is plotted on 2 cycle semi-log. paper.

All specimens are assayed in at least 2 separate batches.

Satisfactory recoveries of added vitamin B_{12} to serum were obtained by the method (Fig. 2).

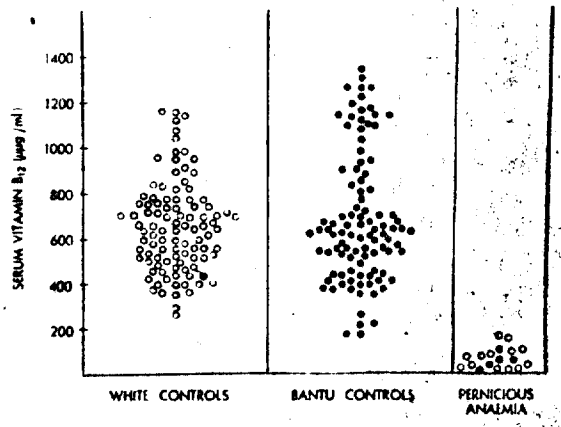

Fig. 3. B_{12} assay results in White and Bantu control subjects and in patients with untreated pernicious anaemia (White patients — open circles: Bantu patients — closed circles).

RESULTS

In the White subjects the mean serum vitamin B_{12} level was 648 $\mu\mu$g./ml., standard deviation (S.D.) 214, and the normal range (mean $\pm$ 2 S.D.) 220-1076 $\mu\mu$g./ml. In the Bantu subjects the mean value was 682 $\mu\mu$g./ml. (S.D. 234), with a range of 214-1150 $\mu\mu$g./ml. Median values were: Bantu 622 $\mu\mu$g./ml., Whites: 640 $\mu\mu$g./ml. In 18 patients with pernicious anaemia in relapse, serum vitamin B_{12} ranged from less than 10 to 160 $\mu\mu$g./ml. (mean 54 $\mu\mu$g./ml.). The results are shown graphically in Fig. 3.

DISCUSSION

While the lower limit of normal of serum vitamin B_{12} content in both White and Bantu subjects is virtually identical, the upper normal limit and mean value in the Bantu are slightly higher than in the White subjects, but the differences are not statistically significant. That these differences are due to the presence of a number of Bantu with relatively high serum vitamin B_{12} levels, is suggested by the fact that the median value in the Bantu is slightly less than that of the White group. Some of the Bantu subjects studied may have been suffering from underlying liver disease, resulting in high serum vitamin B_{12} levels. For practical purposes, the mean value and range of serum vitamin B_{12} concentrations in White and Bantu subjects are similar. We have therefore been unable to confirm the observations of Adams and Forbes [1957] that in the Bantu the mean serum vitamin B_{12} level is very considerably higher than in Whites.

It is worthy of note that the method described allows for clear distinction between controls, and White and Bantu subjects with vitamin B_{12} deficiency (as in pernicious anaemia).

SUMMARY

A method is described for the microbiologic assay of vitamin B_{12} in serum.

Serum vitamin B_{12} levels have been estimated in two groups of equal numbers of Bantu and White subjects. Serum vitamin B_{12} in White subjects ranged from 220-1076 $\mu\mu$g./ml. (mean 648 $\mu\mu$g./ml.), and in the Bantu from 214-1150 $\mu\mu$g./ml. (mean 682 $\mu\mu$g./ml.). These differences are not statistically significant. It is suggested that the slightly higher mean value and upper limit of normal in the Bantu, may be due to the inclusion of a few Bantu subjects with subclinical liver disease.

We wish to thank The Director, South African Institute for Medical Research, for facilities to carry out this study; Glaxo Laboratories, for financial aid to one of us (J.M.); and Dr. A. Zoutendyk, for providing specimens.

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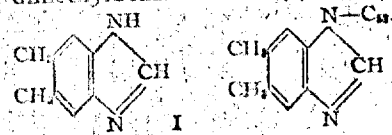
VITAMIN B_{12}. VI. 5,6-DIMETHYLBENZIMIDAZOLE, A DEGRADATION PRODUCT OF VITAMIN B_{12}

Sir:

Degradation of vitamin B_{12} by acid hydrolysis has given a new basic compound which has been identified by its reactions and by synthesis as 5,6-dimethylbenzimidazole (I).



I

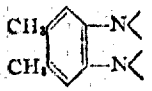
II

The crystalline product melted at 205–206°. *Anal.* Calcd. for $C_9H_{10}N_2$: C, 73.94; H, 6.90; N, 19.17. Found: C, 74.36; H, 6.47; N, 19.21. Potentiometric titration showed an equivalent weight of 144 ± 5; calcd., 146. In 95% ethanol solution in the presence of 0.01 *N* hydrochloric acid, the absorption spectrum of the compound was characterized by maxima at 2745 Å. (E_M7500) and at 2840 Å. (E_M8100). In similar solution in the presence of 0.01 *N* sodium hydroxide, maxima were observed at 2470 Å. (E_M3900), 2775 Å. (E_M 4900), 2810 Å. (E_M5250) and 2880 Å. (E_M5700). The compound was optically inactive. It gave a crystalline picrate, melting point 273–275°. *Anal.* Calcd. for $C_{15}H_{13}N_5O_7$: N, 18.66. Found: N, 18.76.

Treatment of the degradation product with benzoyl chloride in aqueous alkali according to the method of Bamberger and Berlé[1] for the cleavage of benzimidazoles to dibenzamidobenzenes afforded a compound, melting point 262–263°, which was identical with a synthetic sample of the new 4,5-dibenzamido-1,2-dimethylbenzene, melting point 262–262.5° (*Anal.* Calcd. for $C_{22}H_{20}N_2O_2$: C, 76.72; H, 5.85; N, 8.14. Found: C, 76.70; H, 6.01; N, 8.25), prepared by benzoylation of 4,5-diamino-1,2-dimethylbenzene.

The assigned structure of the degradation product was confirmed by the synthesis of 5,6-dimethylbenzimidazole by condensation of 4,5-diamino-1,2-dimethylbenzene with formic acid. The resulting compound had melting point and mixed melting point 204–205°. Its absorption spectrum was identical with that of the natural product, within experimental error. A provisional formula[2] for vitamin B_{12} is represented in II, which is based on the assumption that the dimethylimidazole is terminal and linked to a nitrogen.

It is noted that the 1,2-diamino-4,5-dimethylbenzene moiety (III) appears in 5,6-dimethylbenzimidazole and vitamin B_{12}, and also in riboflavin.



III

Elucidation of the biological implications of this chemical structural relationship will undoubtedly prove of interest.

When a sample of riboflavin was hydrolyzed under the same conditions, a similar isolation technique failed to yield any 5,6-dimethylbenzimidazole.

The authors wish to thank Miss Janice Mayfield for technical assistance, Dr. N. R. Trenner and Mr. R. P. Buhs for the potentiometric titration, and Mr. R. Boos and his associates for the microanalyses.

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NORMAN G. BRINK
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RECEIVED JULY 6, 1949

(1) Bamberger and Berlé, *Ann.*, **273**, 346 (1893).
(2) Brink, *et al.*, THIS JOURNAL, **71**, 1854 (1949).

British Nutrition Society. Brit. J. Nutr. 31(2), 1974

Concentrations or compositions should not be expressed on a percentage basis. The common measurements used in nutritional studies, e.g. digestibility, biological value, and net protein utilization, should be expressed as decimals rather than as percentages, so that amounts of available nutrients can be obtained from analytical results by direct multiplication. See *Metric Units, Conversion Factors and Nomenclature in Nutritional and Food Sciences*, London: The Royal Society, 1972 (paragraph 8).

Nomenclature of Vitamins. Most of the names for vitamins and related compounds that are accepted by the Editors are those recommended by the IUNS Committee on Nomenclature. See *Nutr. Abstr. Rev.*, 1970, 40, 395.

Previous name	Recommended name
Vitamin A_1	Retinol
Retinene or retinal	Retinaldehyde
Vitamin A acid	Retinoic acid
Vitamin A_2 or 3-dehydroretinol	Dehydroretinol
Retinene 2 or 3-dehydroretinal	Dehydroretinaldehyde
Vitamin D_2 or calciferol	Ergocalciferol
Vitamin D_3	Cholecalciferol
Vitamins E	See *Generic descriptors*
Vitamin K_1 or phylloquinone	Phylloquinone
Vitamin K_2 series	*Menaquinones
Vitamin K_3, menadione or menaphthone	Menaphthone
Vitamin B_1, aneurin(e) or thiamine	Thiamin
Vitamine B_2 or riboflavine	Riboflavin
Nicotinic acid or niacin	Nicotinic acid
Niacinamide or nicotinic acid amide	Nicotinamide
Folic acid or folacin(e)	Pteroylmonoglutamic acid
Vitamin B_6, adermin or pyridoxol	Pyridoxine
Pyridoxal	Pyridoxal
Pyridoxamine	Pyridoxamine
Vitamin B_{12} or cobalamin	Cyanocobalamin
Vitamin B_{12a}, B_{12b} or hydroxocobamide	Hydroxocobalamin
Vitamin B_{12c}	Nitritocobalamin
Inositol or *meso*-inositol	*myo*-Inositol
Pantothenic acid	Pantothenic acid
Biotin	Biotin
Choline	Choline
p-Aminobenzoic acid	*p*-Aminobenzoic acid
Vitamin C or L-ascorbic acid	Ascorbic acid
L-dehydroascorbic acid	Dehydroascorbic acid

Generic descriptors. The terms **vitamin A, vitamin C** and **vitamin D** may still be used where appropriate, for example in phrases such as 'vitamin A deficiency', 'vitamin D activity'.

Vitamin E. The term **vitamin E** should be used as the descriptor for all tocol and tocotrienol derivatives exhibiting qualitatively the biological activity of α-tocopherol. The term **tocopherols** should be used as the generic descriptor for all methyl tocols. Thus, the term **tocopherol** is not synonymous with the term **vitamin E.**

Vitamin K. The term **vitamin K** should be used as the generic descriptor for 2-methyl-1,4-naphthoquinone (menaphthone) and all derivatives exhibiting qualitatively the biological activity of phylloquinone (phytylmenaquinone).

Niacin. The term **niacin** should be used as the generic descriptor for pyridine 3-carboxylic acid and derivatives exhibiting qualitatively the biological activity of nicotinamide.

Folic acids. The term **folic acid** may be used to designate the naturally occurring pteroylglutamic acids.

Vitamin B_6. The term **vitamin B_6** should be used as the generic descriptor for all 2-methylpyridine derivatives exhibiting qualitatively the biological activity of pyridoxine.

Vitamin B_{12}. The term **vitamin B_{12}** should be used as the generic descriptor for all corrinoids exhibiting qualitatively the biological activity of cyanocobalamin. The term **corrinoids** should be used as the generic descriptor for all compounds containing the corrin nucleus and thus chemically related to cyanocobalamin. The term **corrinoid** is not synonymous with the term **vitamin B_{12}.**

Amounts of vitamins and summation. All amounts of vitamins should be expressed in terms of their mass rather than in terms of i.u. See *Metric Units, Conversion Factors and Nomenclature in Nutritional and Food Sciences*, London: The Royal Society, 1972 (paras. 8 and 14–20).

Nomenclature of Fatty Acids. In the description of results obtained for the analysis of fatty acids by conventional gas–liquid chromatography, the shorthand designation proposed by Farquhar, J. W., Insull, W., Rosen, P., Stoffel, W. & Ahrens, E. H. (*Nutr. Rev.*, 1959, 17, Supplement) for individual fatty acids should be used in the text, tables and figures. Thus 18:1 should be used to represent a fatty acid with eighteen carbon atoms and one double bond; if the position and configuration of the double bond is unknown, this fatty acid should not be referred to as oleic acid. The shorthand designation should also be used in the synopsis but sentences should be constructed so that it is clear to the non-specialist reader that 18:1 refers to a fatty acid; for example, '...resulted in an increase in the concentration of the fatty acid 18:1 in the liver triglycerides...'. If the positions and configurations of the double bonds are known, and these are important to the discussion, then a fatty acid such as linoleic acid may be referred to as 18:2 Δ 9-*cis*, 12-*cis* (positions of double bonds related to the α-carbon atom). However, when essential and related fatty acids derived from animal tissues are being considered, it is preferable to refer to fatty acids such as linoleic acid as 18:2ω6 and arachidonic acid as 20:4ω6 (position of double bonds related to the ω-carbon atom); it is assumed that the double bonds are methylene-interrupted and are of the *cis*-configuration (see Holman, R. T. in *Progress in the Chemistry of Fats and Other Lipids* Vol. 9, part 1, p. 3. Oxford: Pergamon Press, 1966). Groups of fatty acids that have a common chain length but vary in their double bond content or double bond position should be referred to, for example as C_{20} fatty acids or C_{20} polyunsaturated fatty acids. Impure samples of fatty acids such as those used in the preparation of diets should be referred to, for example as 'linoleic acid'.

Nomenclature of Enzymes. The nomenclature should be that of the Recommendations of the Commission on

* Details of the nomenclature for these and other naturally occurring quinones should follow the Tentative Rules of the IUPAC-IUB Commission on Biochemical Nomenclature (see *Biochem. J.*, 1967, 102, 15).

Inhibiting and Enhancing Effect of Various Chemical Agents on Rat's Resistance to Inoculated Walker 256 Tumor Cells

PETER BUINAUSKAS, M.D., ERIC R. BROWN, Ph.D., and WARREN H. COLE, M.D., *University of Illinois College of Medicine*

It has been reported previously from this laboratory that surgical trauma in the form of celiotomy decreases the rat's resistance to inoculated Walker 256 carcinosarcoma cells.[1-10] Also, anesthesia in all of its modalities has a similar and contributory effect.[5, 11]

Surgeons are aware of the fact that major operative procedures and anesthesia of commensurate duration trigger multiple biologic and physicochemical responses within the body. It is generally assumed that these responses are directed toward repair of the inflicted damage and reestablishment of the normal dynamic biologic state. Therefore it becomes apparent that some of these reparative mechanisms which are necessary for the animal's survival and recovery may also enhance the "takes" of inoculated Walker 256 carcinosarcoma cells. Many of the repair mechanisms are well understood and documented, whereas many others are still obscure.

The series of experiments reported in this paper were carried out with the purpose of shedding some light on the processes triggered by major surgical trauma and the possibility of neutralizing or altering them with various chemical agents. We chose particularly to study the effects of histamine and antihistamine compounds (as discussed further under Discussion) because the former is such a toxic product and is released in the body following stress of numerous types. We were especially interested in the rat's resistance to inoculated tumor graft cells while the responses to surgical trauma and anesthesia were at their peak intensity.

MATERIALS

Animals

White, female, Sprague-Dawley rats weighing 100 to 200 gm. each were used for all of the experiments. Animals were obtained in lots of 100 to 120 and all of the groups used at one time were chosen from the same shipment. Only healthy and vigorous rats were used. They were kept in our laboratory for 48 hours prior to the experiment for evaluation of their physical condition and also to allow them to adjust to our animal hospital diet and environment. Animals were kept in stainless steel cages, 5 in each, in air-conditioned cubicles with the ambient temperature of 72° F.

Diet

The diet consisted of Standard Purina Laboratory Chow pellets and was given ad libitum. Tap water from rubber-stoppered glass dispensers was accessible to the animals at all times.

Chemical Agents

a. **Diphenhydramine Hydrochloride, Parke, Davis:** Benadryl hydrochloride in 30 cc. Steri-Vials containing 10 mg. per cc. of drug with 1:10,000 benzethonium chloride as a germicidal agent. The commerical solution was used undiluted and administered intraperitoneally, 50 mg. per kg. of animal's body weight.

b. **Cyanocobalamin Injection U.S.P.:** Crystalline vitamin B_{12}—Dodex injectable, Organon Inc., West Orange, N. J. The solution contained 100 μg. of cyanocobalamin per ml. of sterile isotonic saline solution containing:

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1.5% benzyl alcohol (preservative), 0.4% sodium chloride, 0.04% sodium acetate and 0.01% glacial acetic acid. The commercial product was used undiluted and administered intraperitoneally, 200 μg. per kg. of animal's body weight.

c. **Dextran of Molecular Weight 77,000-161,000:** Nutritional Biochemicals Corporation, Cleveland, Ohio. The commercial powder was dissolved in sterile physiological saline solution, 100 mg. per ml. and administered intraperitoneally, 1000 mg. per kg. of animal's body weight.

d. **Chlorcyclizine Hydrochloride:** 1-(4-chlorobenzhydryl)-4-methylpiperazine dihydrochloride – Di-Paralene hydrochloride (Abbott); kindly supplied without charge by Abbott Laboratories, North Chicago, Illinois. The dry powder was dissolved in sterile, pyrogen-free distilled water, 5 mg. per ml., and administered intraperitoneally, 50 mg. per kg. of animal's body weight.

e. **Histamine Acid Phosphate:** Nutritional Biochemicals Corporation, Cleveland, Ohio. Each time a fresh solution was prepared by dissolving the dry powder in sterile, pyrogen-free distilled water, calculated 10 mg. of base per ml., and was administered intraperitoneally, 90 mg. of base per kg. of animal's body weight.

f. **Histamine Dihydrochloride:** Nutritional Biochemicals Corporation, Cleveland, Ohio. The solutions were prepared and administered in the same manner and dosage as above under (e).

g. **Promethazine Hydrochloride:** N-(2′ dimethylamino-2′ methyl) ethyl phenothiazine hydrochloride–Phenergan Hydrochloride injection, Wyeth Laboratories, Philadelphia, Pennsylvania. The drug was procured in 10 cc. sterile vials. In addition to active ingredient (25 mg. of promethazine hydrochloride), each cc. contained sodium citrate buffer, 1 mg. sodium formaldehyde sulfoxylate and 5 mg. phenol. The commercial solution was used undiluted and was administered intraperitoneally, 50 mg. of active ingredient per kg. of animal's body weight.

h. **Reserpine U.S.P.:** Commercially available tablets, each containing 1 mg. of active substance, were used for the preparation of solution for injection. Each time a fresh solution, containing 0.1 mg./ml. of reserpine, was prepared in the following manner:

Reserpine	5.0 mg.
Ethanol 95%	2.5 ml.
Ethylene glycol	2.5 ml.
Hydrochloric acid 0.02% up to	50.0 ml.

This solution was administered intraperitoneally, 1.0 mg. of reserpine per kg. of animal's body weight.

i. **Serotonin; 5-Hydroxytryptamine:** Purchased as a complex salt of serotonin creatinine sulfate from Regis Chemical Company, Chicago, Illinois. The dry commercial powder was dissolved in sterile, pyrogen-free water, calculated 3.5 mg. of serotonin per ml., and was administered intraperitoneally, 23.3 mg. of serotonin per kg. of animal's body weight.

j. **Methapyrilene Hydrochloride:** 2-(2-Dimethylamino-ethyl-2-thenylamino) pyridine hydrochloride–Thenylene Hydrochloride (Abbott): kindly supplied without charge by the Abbott Laboratories, North Chicago, Illinois. The dry powder was dissolved in sterile, pyrogen-free distilled water, 5 mg. per ml. and administered intraperitoneally, 50 mg. per kg. of animal's body weight.

k. **Hank's Balanced Salt Solution (BSS):** This solution was prepared from laboratory grade reagents by the method described by Merchant et al.[8] It was buffered to pH 7.4, and was used in the preparation of Walker 256 tumor cell suspensions.

l. **Sodium Chloride Solution, U.S.P.** Solution was procured in multiple dose containers and was used throughout as sterile physiological saline solution. 100 cc. contained 0.9 gm. sodium chloride U.S.P. with methylparaben U.S.P. 0.05 gm. and propylparaben U.S.P. 0.005 gm. as preservatives.

Procedures

a. Preparation of Fresh Walker 256 Tumor Cell Suspension: Donor animals bearing subcutaneous tumors measuring 2 to 3 cm. in diameter were used. Animals were killed and the tumors excised under aseptic conditions. Only solid portions of tumors grossly without evidence of necrosis were used. The glassware and instruments used for the preparations and "aging" of cell suspensions were chemically clean and sterilized just before usage. Approximately 5 gm. of excised tumor tissue was placed on a sterile glass shell, and, using sharp scissors, was subdivided minutely. To this, small amounts of Hank's Balanced Salt Solution (BSS) were added to make it more workable. This mince was then diluted to 50 per cent and placed in an all glass Ten Broeck tissue mill. Tumor tissue was ground in the mill and turbid supernatant containing cells was decanted and filtered through folded sterile surgical gauze by gravity into a sterile flask containing a sterile magnetic stirring rod.

Additional sterile BSS was poured through the filter gauze until 20% suspension was obtained. An aliquot of suspension was aseptically taken from the flask for microscopic inspection and preliminary count of tumor cells.

The "virulence" of this tumor as evidenced by production of "takes" after injection of various numbers of cells per cubic millimeter of suspending medium has varied considerably and, probably, in part is influenced by the immunochemical responses of animals through which it is being serially passed during maintenance.

b. "Aging" of Tumor Cells: At the time of these experiments inoculation of fresh BSS suspension of 2000 tumor cells usually resulted in "takes" in 95 to 100 per cent of the experimental animals. In order to determine whether a given procedure or chemical agent will increase or decrease the percentage of takes of tumor cells, it is necessary that the takes in control animals should be between 35 and 65 per cent. Other workers in this laboratory[2] have shown that aging of cells in suspension will reduce the number of takes.

To achieve more controlled conditions, the aging of cell suspensions in these experiments was carried out not at room temperature but at 6° C. ± 0.05. By preliminary experiments it was found that inoculations of 2000 tumor cells aged at the above temperature for 24 hours will give the desired range of takes in controls.

The flask with freshly prepared suspension as under (a) above was stoppered, carried into a 6° C. cold room and placed on a magnetic stirrer. The suspension was gently stirred during the entire 24 hours of aging. Vigorous stirring was avoided to prevent aeration and incorporation of CO_2.

At the end of the aging period viable cells were counted again and a final tumor cell suspension in BSS, containing 2000 cells per ml., was prepared by serial dilution in amounts sufficient for inoculation of all animals for the experiment. Each animal was inoculated with 2000 tumor cells in 1 ml. of Hank's Balanced Salt Solution subcutaneously on the back.

c. Counting of Tumor Cells: Aliquots of concentrated tumor cell suspension were removed aseptically before and after aging and counted in a hemocytometer using eosin 1:2000 solution as diluent. Only the cells that did not stain pink with eosin were regarded as viable and counted. Two counts by three competent persons were made each time and the arithmetical mean value of combined counts was used for calculations for serial dilutions. Usually one of the serial dilutions was also counted in the same manner.

METHODS

Preparation of Animals

In the evening prior to the experimental day, the animals were shaved closely on their backs and abdomens by electric clippers. Extreme care was taken to insure that the skin was not injured during this procedure. Shaven backs facilitated aseptic inoculation of the tumor cells and also the discovery of the first appearance of tumors.

The shaven abdomens were used for performing celiotomies and/or for injection of drugs intraperitoneally.

Anesthesia

All animals undergoing celiotomy were anesthetized by intraperitoneal injection of pentobarbital sodium, 30 mg. per kg. of body weight.

Technique of Celiotomy

The anesthetized animals were secured to the operating board with rubber bands on all four limbs, avoiding stretching the animals too tightly as this interfered with respiration and tended to produce casualties. The skin was then aseptically prepared using tincture of iodine and 75% ethanol. The abdomens were opened in rapid succession by means of a vertical midline incision. This extended from the xiphoid process to the symphysis pubis. All abdomens were incised along the linea alba, to minimize bleeding, and peritoneum was opened along the same line. The abdominal wall was draped along the sides of the incision with sterile gauze dressing moistened in sterile physiological saline solution. The abdominal contents consisting of small and large intestines, spleen and liver were then delivered through the incision and placed on the drapes.

The abdominal viscera were left exposed in this manner for 45 minutes. During this period the position of viscera on the drapes was changed every 15 minutes in order to subject them to the trauma of handling. Also, at 10 minute intervals, all exposed organs were sprinkled with sterile physiological solution to prevent drying. At the end of 45 minutes,

the abdominal contents were replaced in the peritoneal cavity and the incision in the abdominal wall was closed in layers, using a continuous suture of #60 cotton.

No eviscerations or wound infections were noted among the animals in this series of experiments.

Inoculation of Tumor Cells

Upon completion of celiotomies, all animals for the day's experiment (those operated upon and animals that did not undergo surgery) were inoculated at random with Walker 256 tumor cell suspension, 1 ml. of HBSS containing 2000 cells, aged for 24 hours, on their backs and were placed in a large container where they could mix freely.

After completion of the inoculation of tumor cells, the animals were picked at random from this container and divided into groups of 10 animals for further treatment as summarized in Table 1. Animals that died because of anesthesia or in the process of celiotomy were excluded from the experiment (3 per cent). The animals that died within the period of time between the celiotomy or treatment and the appearance of tumor at the site of inoculation were rejected from the experiment in the same manner (2 per cent). All other animals that survived were examined daily during the first 72 hours, and twice a week thereafter.

First palpable tumors were usually found 14 days after inoculation of tumor cell suspension. These observations were continued until no

Table 1. Summary of Experiments

Group	Chemical Agents, Dose and Route of Administration	Total No. of Animals	Total No. of Takes	Per cent Takes
1	Diphenhydramine hydrochloride (Benadryl) 50 mg. per kg. body weight, I.P.	29	17	58.6
2	Cyanocobalamin Injection U.S.P. (Vit. B_{12}) 200 μg. per kg. body weight, I.P.	30	17	56.6
3	Dextran Mol. Wt. 77,000 – 161,000 1000 mg. per kg. body weight, I.P.	30	14	46.6
4	Di-Paralene hydrochloride 50 mg. per kg. body weight, I.P.	30	22	73.3
5	Histamine acid phosphate 90 mg. of base per kg. body weight, I.P.	30	16	53.0
6	Histamine dihydrochloride 90 mg. of base per kg. body weight, I.P.	36	20	55.5
7	Promethazine (Phenergan) 50 mg. per kg. body weight, I.P.	30	18	60.0
8	Reserpine U.S.P. 1.0 mg. per kg. body weight, I.P.	60	32	53.3
9	Serotonin (5-hydroxytryptamine) 23.3 mg. of base per kg. body weight, I.P.	30	22	73.3
10	Methapyrilene HCl (Thenylene HCl) 50 mg. per kg. body weight, I.P.	29	24	82.6
11	Celiotomy followed by intraperitoneal injection of Benadryl, 50 mg. per kg. body weight	29	22	75.8
12	Celiotomy followed by I.P. injection of Di-Paralene hydrochloride, 50 mg. per kg. body weight	28	23	82.1
13	Celiotomy followed by intraperitoneal injection of Phenergan, 50 mg. per kg. body weight	29	24	82.7
14	Celiotomy followed by intraperitoneal injection of reserpine, 1.0 mg. per kg. body weight	57	34	57.0
15	Celiotomy followed by I.P. injection of Thenylene hydrochloride, 50 mg. per kg. of body weight	28	25	89.2
16	Celiotomy	58	44	75.8
17	Controls: Sterile physiological saline solution, 2.0 ml. injected intraperitoneally	70	34	48.5

new takes were found for two consecutive weeks. The average time for this period was 6 weeks.

A "take" was considered the establishment of a solid subcutaneous mass at the site of inoculated tumor cells. This mass increased progressively in size, became fluctuant in the center and occasionally reached enormous proportions, covering the entire back of the animal. At the end of the observation period all animals were killed. The clinically apparent tumors were excised, sectioned and inspected. Representative tumor samples were taken from the entire experimental group for histological examination. During this entire series the tumors in experimental animals did not differ from the original tumor used for the preparation of cell suspension, and remained on histological examination a carcinosarcoma.

The clinical findings and counts of takes coincided accurately with autopsy findings. No abscesses, hematomas or unrelated tumors were found at the sites of inoculation of tumor cell suspension.

Administration of Drugs

After all animals were inoculated with tumor cells, as stated previously, they were picked at random from the container and divided into groups (see Table 1) for further treatment as follows:

GROUP 1. Each animal in this group received diphenhydramine hydrochloride, 50 mg. per kg. of body weight, intraperitoneally.

GROUP 2. Each animal in this group received cyanocobalamin (crystalline vitamin B_{12}), 200 μg. per kg. of body weight, intraperitoneally.

GROUP 3. Each animal in this group received dextran, molecular weight of 77,000–161,000, 1000 mg. per kg. of body weight, intraperitoneally.

GROUP 4. Each animal in this group received chlorcyclizine hydrochloride (Di-Paralene hydrochloride), 50 mg. per kg. of body weight, intraperitoneally.

GROUP 5. Each animal in this group received histamine acid phosphate, 90 mg. of base per kg. of body weight intraperitoneally.

GROUP 6. Each animal in this group received histamine dihydrochloride, 90 mg. of base per kg. of body weight intraperitoneally.

GROUP 7. Each animal in this group was given promethazine hydrochloride (Phenergan hydrochloride), 50 mg. per kg. of body weight, intraperitoneally.

GROUP 8. Each animal in this group was given reserpine U.S.P., 1 mg. per kg. of body weight, intraperitoneally.

GROUP 9. Each animal in this group was given 5-hydroxytryptamine (serotonin), 23.3 mg. per kg. of body weight, intraperitoneally.

GROUP 10. Each animal in this group was given methapyrilene hydrochloride (Thenylene hydrochloride), 50 mg. per kg. of body weight, intraperitoneally.

GROUP 11. Each animal in this group was first subjected to celiotomy, then given diphenhydramine hydrochloride, 50 mg. per kg. of body weight, intraperitoneally.

GROUP 12. Each animal in this group was first subjected to celiotomy, then given chlorcyclizine hydrochloride, 50 mg. per kg. of body weight intraperitoneally.

GROUP 13. Each animal in this group was first subjected to celiotomy, then given promethazine hydrochloride, 50 mg. per kg. of body weight, intraperitoneally.

GROUP 14. Each animal in this group was first subjected to celiotomy, then was given reserpine 1 mg. per kg. of body weight, intraperitoneally.

GROUP 15. Each animal in this group was first subjected to celiotomy, then was given methapyrilene hydrochloride, 50 mg. per kg. of body weight, intraperitoneally.

GROUP 16. Each animal in this group underwent celiotomy under pentobarbital sodium anesthesia, 30 mg. per kg. of body weight, intraperitoneally.

GROUP 17. Each animal in this group was given sodium chloride injection, U.S.P., 2 ml., intraperitoneally.

RESULTS

The results of all the experiments are summarized in Table 1. The statistical analysis of the experiments is depicted in Tables 2, 3 and 4.

Groups 1 through 10 demonstrate the effect of various chemical agents, injected intraperitoneally, on takes of Walker 256 carcinosarcoma cells, aged for 24 hours and inoculated subcutaneously.

All control animals, which were treated only with saline solution given intraperitoneally, were combined and tabulated as Group 17.

Inspection of data for groups 1 to 10, inclusive, and comparison of these with controls (Group 17) reveals the following:

1. There were 17 takes among 29 diphenhydramine hydrochloride treated animals

Table 2. Effect of Various Chemical Agents on Takes in Rats Compared with the Takes in Control Rats Injected with Physiological Saline

Group	Agent	Per cent Takes in Controls (Saline)	Per cent Takes in Group	Apparent Effect in Per cent	X^2 Value	P Value
1	Diphenhydramine hydrochloride	48.5	58.6	+ 10.1	2.474	< 0.30
2	Cyanocobalamin (Vit. B_{12})	48.5	58.6	+ 8.1	0.274	< 0.50
3	Dextran	48.5	46.6	- 1.9	--	--
4	Chlorcyclizine hydrochloride	48.5	73.3	+ 24.8	6.278	< 0.02
5	Histamine acid phosphate	48.5	53.0	+ 4.5	0.040	< 0.80
6	Histamine dihydrochloride	48.5	55.5	+ 7.0	0.226	< 0.50
7	Promethazine hydrochloride	48.5	60.0	+ 11.5	0.688	< 0.30
8	Reserpine, U.S.P.	48.5	53.3	+ 4.8	0.040	< 0.80
9	Serotonin	48.5	73.3	+ 24.8	6.278	< 0.02
10	Methapyrilene hydrochloride	48.5	82.6	+ 34.1	11.352	< 0.001

Table 3. Effect of Various Chemical Agents Administered to Rats Subjected to Celiotomy as Compared with Control Animals Having Celiotomy Only

Group	Agent	Per cent Takes in Celiotomy Only	Per cent Takes in Group	Apparent Effect in Per cent	X^2 Value	P Value
11	Diphenhydramine hydrochloride	75.8	75.8	± 0	--	--
12	Chlorcyclizine hydrochloride	75.8	82.1	+ 6.3	0.207	<0.50
13	Promethazine hydrochloride	75.8	82.7	+ 6.9	0.207	<0.50
14	Reserpine, U.S.P.	75.8	57.0	-18.8	4.252	<0.05
15	Methapyrilene hydrochloride	75.8	89.2	+ 13.4	1.382	<0.30

Table 4. Effect of Various Chemical Agents on Takes in Rats Having Celiotomy Compared with the Takes in Rats Injected with Physiological Saline Alone

Group	Agent	Per cent Takes in Saline Controls	Per cent Takes in Group	Apparent Effect in Per cent	X^2 Value	P Value
11	Diphenhydramine hydrochloride	48.5	75.8	+ 27.3	5.144	0.02
12	Chlorcyclizine hydrochloride	48.5	82.1	+ 33.8	7.934	<0.01
13	Promethazine hydrochloride	48.5	82.7	+ 34.2	8.505	<0.01
14	Reserpine, U.S.P.	48.5	57.0	+ 8.5	1.133	0.30
15	Methapyrilene hydrochloride	48.5	89.2	+ 40.7	11.595	<0.001
16	Celiotomy only	48.5	75.8	+ 27.3	5.144	0.02

(58.6%) as compared with controls in which there were 34 takes among 70 animals (48.5%). Apparent enhancement of takes: 10.1%.

2. There were 17 takes among 30 cyanocobalamin treated animals (56.6%) as compared with controls in which there were 34 takes among 70 animals (48.5%). Apparent enhancement of takes: 8.1%.

3. There were 14 takes among 30 dextran treated animals (46.6%) as compared with controls in which there were 34 takes among 70 animals (48.5%). Apparent enhancement of takes: −1.9%.

4. There were 22 takes among 30 chlorcyclizine hydrochloride treated animals (73.3%) as compared with controls in which there were 34 takes among 70 animals (48.5%). Apparent enhancement of takes: 24.8%.

5. There were 16 takes among 30 histamine acid phosphate treated animals (53.0%) as compared with controls in which there 34 takes among 70 animals (48.5%). Apparent enhancement of takes: 4.5%.

6. There were 20 takes among 36 histamine dihydrochloride treated animals (55.5%) as compared with controls in which there were 34 takes among 70 animals (48.5%). Apparent enhancement of takes: 7.0%.

7. There were 18 takes among 30 promethazine hydrochloride treated animals (60.0%) as compared with controls in which there were 34 takes among 70 animals (48.5%). Apparent enhancement of takes: 11.5%.

8. There were 32 "takes" among 60 reserpine treated animals (53.3%) as compared with controls in which there were 34 takes among 70 animals (48.5%). Apparent enhancement of takes: 4.8%.

9. There were 22 takes among 30 5-hydroxytryptamine treated animals (73.3%) as compared with controls in which there were 34 takes among 70 animals (48.5%). Apparent enhancement of takes: 24.8%.

10. There were 24 takes among 29 methapyrilene hydrochloride treated animals (82.6%) as compared with controls in which there were 34 takes among 70 animals (48.5%). Apparent enhancement of takes: 34.1%.

Groups 11 through 15 consisted of the animals that were first subjected to celiotomy and shortly thereafter were treated with various agents. Animals in Group 16 were subjected to celiotomy only prior to inoculation of tumor cells.

Inspection of data for Groups 11 to 15, inclusive, and comparison of these with those in Group 16, reveal the following from the standpoint of enhancement of takes or neutralization of the decreased resistance.

11. There were 22 takes among 29 animals subjected to celiotomy and thereafter treated with diphenhydramine hydrochloride (75.8%) as compared with celiotomy-only controls, in which there were 44 takes among 58 animals (75.8%). Apparent enhancement of takes: 0%.

12. There were 23 takes among 28 animals subjected to celiotomy and shortly thereafter treated with chlorcyclizine hydrochloride (82.1%) as compared with celiotomy-only controls, in which there were 44 takes among 58 animals (75.8%). Apparent enhancement of takes: 6.3%.

13. There were 24 takes among 29 animals subjected to celiotomy and shortly thereafter treated with promethazine hydrochloride (82.7%) as compared with celiotomy-only controls, in which there were 44 takes among 58 animals (75.8%). Apparent enhancement of takes: 6.9%.

14. There were 34 takes among 57 animals subjected to celiotomy and shortly thereafter treated with reserpine (57.0%) as compared with celiotomy-only controls, in which there were 44 takes among 58 animals (75.8%). Apparent reduction of takes: 18.8% (which may perhaps be classified as neutralization of decreased resistance).

15. There were 25 takes among 28 animals subjected to celiotomy and shortly thereafter treated with methapyrilene hydrochloride (89.2%) as compared with celiotomy-only controls, in which there were 44 takes among 58 animals (75.8%). Apparent enhancement of takes: 14.4%.

Finally, completing the survey, inspection of data for Groups 11-16, inclusive, and comparison of these with those in Group 17 (injected with sodium chloride) reveals the following:

11. There were 22 takes among 29 animals subjected to celiotomy and then treated with diphenhydramine hydrochloride (75.8%), as compared to 34 takes among 70 animals treated with physiological saline (48.5%). Enhancement of takes: 27.3%.

12. There were 23 takes among 28 animals subjected to celiotomy and then treated with chlorcyclizine hydrochloride (82.1%), as compared to 34 takes among 70 animals treated with physiological saline (48.5%). Enhancement of takes: 33.8%.

13. There were 24 takes among 29 animals subjected to celiotomy and then treated with promethazine hydrochloride (82.7%), as com-

pared to 34 takes among 70 animals treated with physiological saline (48.5%). Enhancement of takes: 34.2%.

14. There were 34 takes among 57 animals subjected to celiotomy and then treated with reserpine (57.0%), as compared to 34 takes among 70 animals treated with physiological saline (48.5%). Enhancement of takes: 8.5%.

15. There were 25 takes among 28 animals subjected to celiotomy and then treated with methapyrilene hydrochloride (89.2%), as compared to 34 takes among 70 animals treated with physiological saline (48.5%). Enhancement of takes: 40.7%.

16. There were 44 takes among 58 animals subjected to celiotomy only (75.8%), as compared to 34 takes among 70 animals treated with physiological saline (48.5%). Enhancement of takes: 27.3%.

DISCUSSION

As summarized by Pelletier and colleagues,[9] numerous investigators have shown that tumors in human beings and laboratory animals contain antihistamine substances. For this reason, the following antihistaminics were evaluated for their enhancing effects on tumor "takes": promethazine HCl; diphenydramine HCl; methapyrilene HCl; chlorcyclizine HCl. These substances, as shown in the table, enhanced tumor takes in laboratory animals. This would confirm the findings of Pelletier that the antihistamine effect of tumors plays a role in the increase of tumor takes. It would also indicate that histamine does not play a major role in increasing tumor takes in rats, and this is shown in the tables by the role of histamine PO_4 and histamine HCl, which did not significantly increase the number of takes. However, when 5-hydroxytryptamine was used a significant increase in takes was seen. The antagonist to 5-hydroxytryptamine, reserpine, decreases the takes in celiotomized animals inoculated with Walker 256 carcinosarcoma. According to Kabat and Mayer[6] the rat is extremely refractory to histamine whereas 5-hydroxytryptamine appears to play a more important role, and in these species antihistamines are ineffective, but antagonists of serotonin block the reaction.

In the rat, dextran causes the release of endogenous histamine.[3] When dextran was administered to rats inoculated with the Walker 256 cells there was no enhancement whatsoever of takes. This would again imply that histamine whether endogenous or administered plays no role in tumor takes in the rat. On the other hand, the fact that antihistaminics enhanced takes would indicate that, instead of histamine, it is the antihistaminics that have the important role.

Fisher and Fisher[1] indicated that, during the process of celiotomy, hepatic damage results from the operation. The regenerating liver has a stimulating effect on tumor takes through the production of unknown humoral factors. Kato and Murakami[7] demonstrated that, when an animal's liver was damaged by carbon tetrachloride, vitamin B_{12} was released into the system by the liver. For this reason cyanocobalamin was tested for its effect on tumor takes. As shown in the tables, it has a slight enhancing effect.

A brief summary of the effect of each substance tested is given below. The figures in parentheses indicate percentage of increase (+) or decrease (–):

1. Dextran (–1.9%), histamine acid phosphate (+4.5%), histamine dihydrochloride (+7.0%), reserpine (+4.8%), promethazine hydrochloride (+11.5%) and diphenhydramine hydrochloride (+10.1%) when injected alone did not significantly influence takes as compared to the takes in controls treated with physiological saline.

2. Methapyrilene hydrochloride, 5-hydroxytryptamine and chlorcyclizine increased takes by 34.1%, 24.8% and 24.8% respectively as compared to the takes in controls treated with physiological saline.

3. Celiotomy alone significantly enhanced takes (+27.3%) as compared to the takes in controls treated with physiological saline.

4. Diphenhydramine hydrochloride (0%), chlorcyclizine (+6.3%) and promethazine (+6.9%) when used in conjunction with celiotomy did not significantly influence takes as compared to takes in animals subjected to celiotomy alone.

5. Methapyrilene hydrochloride increased takes by +14.4% as compared to takes in animals subjected to celiotomy alone.

6. Reserpine decreased takes by –18.8% as compared to takes in animals subjected to celiotomy alone.

7. In animals subjected to celiotomy, and, in addition to this, treated with various chemical agents, the following was noted:

a. Diphenhydramine hydrochloride did not enhance the takes beyond the enhancement of 27.3% which was the result of celiotomy alone as compared to saline treated controls.

b. In animals treated with chlorcyclizine hydrochloride, the enhancement of takes was +33.8% as compared to controls treated with physiological saline, or +6.5% above the enhancement of +27.3% which was the result of the celiotomy alone.

c. In animals subjected to celiotomy and then treated with promethazine hydrochloride there was enhancement of +34.2% as compared with control animals treated with physiological saline, or + 6.9% above the enhancement of 27.3% which was the result of celiotomy alone.

d. In animals treated with reserpine, there was enhancement of only +8.5% of takes as compared to the takes in controls treated with physiological saline, or a *decrease* of −18.8% below the percentage of enhancement produced by celiotomy only.

e. In animals treated with methapyrilene hydrochloride there was +40.7% enhancement of takes above takes in controls treated with physiological saline, or an increase of +13.4% above enhancement of takes produced by celiotomy alone (27.3%).

SUMMARY

In our experiments, histamine did not play a major role in increasing the number of tumor takes in rats inoculated with an approximately 50% take level of Walker 256 cells. Conversely, antihistamines were found to cause a marked enhancement of takes, confirming the fact that tumor substances contain antihistaminics which are important in the enhancement of Walker 256 tumor in rats. Vitamin B_{12} may have a role in enhancement of takes in this experimental system. Reserpine, the serotonin antagonist, had significant effect in decreasing tumor takes.

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MICROBIAL DEGRADATION OF CORRINOIDS

III. PIGMENTS DERIVED FROM VITAMIN B_{12} BY *PSEUDOMONAS RUBESCENS*

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ABSTRACT

BURGUS, R. C. (Wayne State University, Detroit, Mich.), J. B. HUFHAM, W. M. SCOTT, AND J. J. PFIFFNER. Microbial degradation of corrinoids. III. Pigments derived from vitamin B_{12} by *Pseudomonas rubescens*. J. Bacteriol. 88:1139-1144. 1964.—Products derived from vitamin B_{12} by *Pseudomonas rubescens* under anaerobic conditions were examined. After incubation of the organism in broth containing Co^{57}- or P^{32}-vitamin B_{12}, electrophoresis of the extracted corrinoids yielded two major, yellow, radioactive fractions, designated A and B, with spectral and electrophoretic properties similar to pigments I and II, derived from vitamin B_{12} by *Aerobacter aerogenes*. Fractions A and B were essentially inactive in promoting the growth of *Lactobacillus leichmannii*. Chromatography on carboxymethylcellulose separated both fractions A and B into four yellow, radioactive fractions. The absorption spectrum of each of the major subfractions showed a maximum in the ultraviolet region characteristic of a 5,6-dimethylbenzimidazole nucleotide, but lacked a maximum in the 360-mμ region characteristic of vitamin B_{12} and many of its analogues and derivatives. The pigments were stable to cyanide and, although they were more stable to air and light than were the vitamin B_{12} coenzymes and coenzyme analogues, they were apparently slowly decomposed by light. The data suggest that the bacteria alter the corrin nucleus of vitamin B_{12}.

During our investigation of the catabolism of vitamin B_{12}, we found that a number of bacteria form new colored products from the vitamin, including two yellow pigment fractions which can be easily separated from known corrinoids (Scott et al., 1964). Of the bacteria studied, *Pseudomonas rubescens* (Pivnick, 1955) produces these two yellow fractions the most rapidly and in the highest yields.

While these investigations were in progress, the conversion of vitamin B_{12} to brownish-yellow pigments by a strain of *Aerobacter aerogenes* was reported by Helgeland, Jonsen, and Laland (1961), who designated the two pigments produced most abundantly pigments I and II. More recently, they reported that pigment I, although essentially inactive in promoting the growth of *Ochramonas malhamensis*, *Lactobacillus leichmannii*, or *Euglena gracillis*, showed some antipernicious anemia activity in man (Helgeland et al., 1963). The nature of pigments derived from vitamin B_{12} by bacteria is therefore of increased interest because, as these authors pointed out, all of the known substances with antipernicious anemia activity have also shown microbiological activity.

This report is concerned with the fractionation and preliminary characterization of the yellow pigments derived from vitamin B_{12} by *P. rubescens*.

MATERIALS AND METHODS

Cultural conditions. P. rubescens (ATCC 12099) was grown at 30 C in nutrient broth (Difco) containing 1% glucose.

To demonstrate the effect of a culture of *P. rubescens* on Co^{57}-vitamin B_{12}, a stock solution containing 1.0 mg of Co^{57}-vitamin B_{12} (4,000 counts per min) in water was autoclaved and added aseptically to 10 ml of sterile broth in a test tube. The tube was inoculated, incubated aerobically for 24 hr to obtain growth, and then incubated anaerobically for 39 days. An uninoculated control tube was included.

In another small-scale experiment, the culture contained 2.5 mg of P^{32}-vitamin B_{12} (1,700 counts per min) in 10 ml of medium. This culture was incubated for 24 hr aerobically and 9 days anaerobically.

To obtain larger amounts of Co^{57}-labeled degradation products, 500 ml of medium were prepared by adding an autoclaved stock solution containing 50 mg of Co^{57}-vitamin B_{12} (35,000 counts per min) to sterile medium in a 2-liter flask. The medium was inoculated with a 10-ml, 24-hr culture, shaken aerobically for 24 hr, sealed with Parafilm, and then incubated anaerobically for 13 days.

Fractionation of corrinoids. After incubation, each culture was centrifuged to remove the cells. The supernatant fluid was concentrated to 0.1 volume at 40 C under vacuum, saturated with ammonium sulfate, made 0.001 M in potassium cyanide, and extracted three times with 2-volume portions of benzyl alcohol. To the combined benzyl alcohol extracts were added 4 volumes of diethyl ether, and the mixture was extracted with three 0.1-volume portions of 0.001 M potassium cyanide. The combined aqueous extracts, containing the corrinoids, were then concentrated and lyophilized.

The corrinoids from small-scale experiments were first separated from contaminating salts by washing the lyophilized extracts with cold, dry methanol, and then were subjected to paper electrophoresis. The methanolic solutions were applied to strips (1 by 35 cm) of Whatman no. 1 paper, or to strips (2.5 by 35 cm) of Whatman 3 MM paper, depending on the size of the sample. Separations usually took 2 to 4 hr at 500 v in 1 N acetic acid-0.001 M potassium cyanide buffer, or 0.5 M sodium phosphate buffer (pH 6.5)-0.001 M potassium cyanide buffer.

For separations of larger quantities of corrinoids, electrophoresis was carried out on a column (1.2 by 55 cm) of Whatman standard-grade cellulose in the acetic acid-cyanide buffer. The methanol extraction was found to be unnecessary for good separation in column electrophoresis, so this step was omitted. A solution of the lyophilized corrinoid extract in buffer was applied and, after 18 to 20 hr of electrophoresis at 500 v, the fractions were removed from the column by elution with 1 N acetic acid. The yellow-pigment fractions were subjected to repeated electrophoresis; usually three separations were required to obtain each fraction as a single zone.

The fractions separated by electrophoresis were purified further by ion-exchange chromatography. Bio-Rad Cellex CM carboxymethylcellulose (Calbiochem) was washed several times with 1 N hydrochloric acid and adjusted to pH 3.8 with 0.001 N acetic acid. Samples were applied to columns (0.2 by 10 to 15 cm) in water or in 0.001 N acetic acid solution, and columns were developed with water or with acetic acid solutions of increasing concentrations (0.001 to 1.0 N).

Radiotracers. Co^{57}-vitamin B_{12} was obtained as Racobalamin (Abbott Laboratories, North Chicago, Ill.). P^{32}-vitamin B_{12} was prepared biosynthetically from $Na_2HP^{32}O_4$. The synthesis was carried out according to the method described by Speedie and Hull (1960) for the preparation of unlabeled vitamin, with the use of *Propionibacterium freudenreichii*, with $Na_2HP^{32}O_4$ added to the medium. No attempt was made to synthesize larger quantities for additional experiments, since the specific activity of the P^{32}-labeled vitamin obtained by this method was very low. Samples (1.0 ml) of radioactive solutions were dried in stainless-steel planchets (1 by $\frac{5}{32}$ in.; 2.54 by 0.39 cm) and counted in a thin-window, gas-flow, proportional counter. For radioautography, paper electrophoresis strips were taped to strips (2 by 25 cm) of Kodak No Screen X-ray film, and the films were developed after 5 to 10 days of exposure.

Absorption spectra. Spectra were obtained by use of 1.0-cm cells in a Beckman model DB recording spectrophotometer. Appropriate blanks were placed in the reference beam to correct for the absorbance of the solvents.

RESULTS

P. rubescens derived radioactive products from Co^{57}-vitamin B_{12} which were not produced in significant quantities in the uninoculated medium, even after prolonged incubation (39 days; Fig. 1). The yellow, radioactive fraction on strip 1, electronegative with respect to vitamin B_{12}, usually occurred in very low yields or was absent with less-prolonged incubation. Not visible on the radioautograph is a labeled fraction with a mobility similar to that of factor B, which usually occurred as a pink zone on electrophoresis strips from *P. rubescens* cultures. Other than the red fraction with a mobility similar to that of vitamin B_{12}, the only Co^{57}-labeled fractions occurring in significant yields were the yellow ones marked A and B on the radioautograph.

When the culture was incubated in the dark and the pigment fractions were isolated under subdued red light, fractions A and B were ob-

tained in yields comparable to those obtained in the light. Thus, light is not required for the formation of the pigments from vitamin B_{12}.

The radioautograph of an electrophoresis strip of the corrinoids from the culture containing P^{32}-vitamin B_{12} showed faintly visible spots corresponding to A and B, but the darkening was not intense enough to photograph.

Fractionation of Co^{57}-labeled yellow pigments. The 500-ml culture of *P. rubescens* containing Co^{57}-vitamin B_{12} was yellow-brown after 5 days of incubation, and aeration failed to return the original red color. After the culture was incubated for an additional 8 days, column electrophoresis of the extracted corrinoids yielded several radioactive, colored fractions. Some of the properties of the major fractions are shown in Table 1. The minor pink fraction, similar in mobility to factor B, was not studied further in these experiments. Helgeland et al. (1961) identified a similar pink fraction, derived from vitamin B_{12} by *A. aerogenes*, as aquocobalamin. The major red fraction was assumed to be recovered vitamin B_{12} on the basis of its electrophoretic mobilities and its absorption spectrum. The yellow fractions, A and B, had

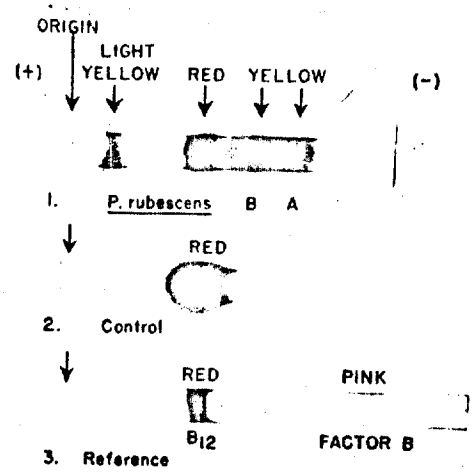

FIG. 1. *Radioautographs of electrophoresis strips of corrinoids from a Pseudomonas rubescens culture (1) and an uninoculated control (2). The locations of vitamin B_{12} and factor B are depicted in a photograph (3) of a reference electrophoresis strip. Electrophoresis was carried out at 500 v for 2.5 hr on Whatman no. 1 paper in acetic acid-cyanide buffer. The colors of the zones are shown above the strips.*

TABLE 1. *Major column electrophoresis fractions from a Pseudomonas rubescens culture containing Co^{57}-vitamin B_{12}.**

Color	Co^{57} activity (counts per min)	Per cent original activity	Electrophoretic mobility†		
			Acetic acid-cyanide buffer		Phosphate-cyanide buffer (pH 6.5)
			Relative to vitamin B_{12}	Relative to factor B	Relative to vitamin B_{12}
Pink	260	1	2.0	1.00	—
Yellow-orange (fraction A)	3,220	9	1.8	0.85	Slightly electronegative
Yellow (fraction B)	3,880	11	1.4	0.70	Slightly electronegative
Red	3,120	9	1.0	0.50	Same as vitamin B_{12}

* The Co^{57}-vitamin B_{12} activity added to the culture was 35,000 counts per min. A total of 24,200 counts per min of Co^{57} activity was recovered from the electrophoresis column fractions listed in the table and in intermediate fractions.

† Electrophoretic mobilities were determined on Whatman 3 MM paper. Electrophoresis was carried out at 500 v for 3 hr. Figures denote relative rates of migration to the cathode.

electropositive mobilities greater than vitamin B_{12} and less than factor B in acetic acid-cyanide buffer. Both fractions were slightly electronegative with respect to vitamin B_{12} in phosphate buffer at pH 6.5, but they were not well separated from each other, or from vitamin B_{12}, in that system.

Column fractions A and B were further purified by paper electrophoresis, first in phosphate-cyanide buffer, and then in acetic acid-cyanide buffer. Both in water and in 0.1 M potassium cyanide, the spectra of fractions A and B (Fig. 2 and 3) are very similar to the spectra reported by Helgeland et al. (1961) for their pigments I and II, respectively.

When the 0.1 M cyanide solution of fraction A was acidified with acetic acid and aerated, the

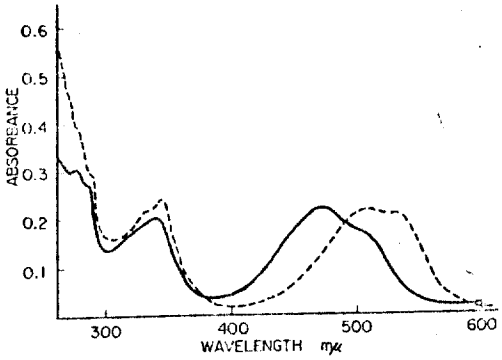

FIG. 2. *Absorption spectra of electrophoresis column fraction A, 10 ml of solution diluted 1:10, in water* (——), *and in 0.1 M potassium cyanide* (- - - -).

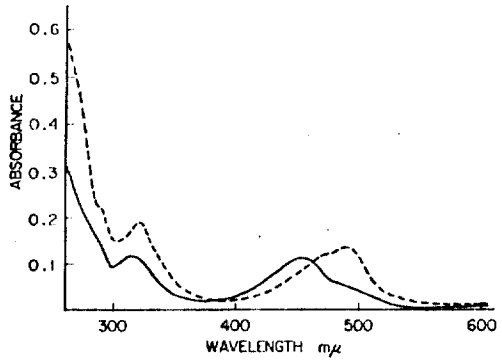

FIG. 3. *Absorption spectra of electrophoresis column fraction B, 10 ml of solution diluted 1:10, in water* (——), *and in 0.1 M potassium cyanide* (- - - -).

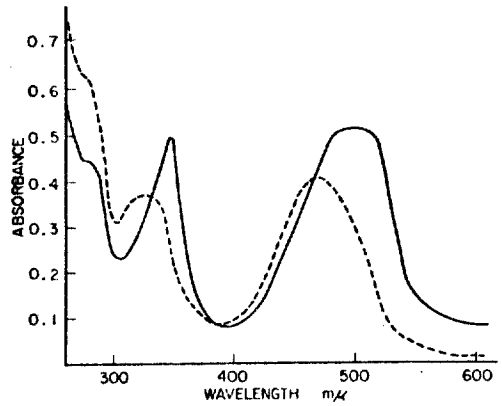

FIG. 4. *Absorption spectra of carboxymethylcellulose column fractions A-1* (——) *and A-2* (- - - -), *in water. Concentrations not determined.*

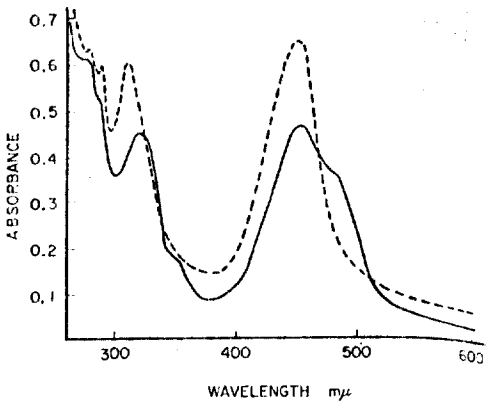

FIG. 5. *Absorption spectra of carboxymethyl cellulose column fractions B-1* (——) *and B-2* (- - - -), *in water. Concentrations not determined.*

color of the solution changed from red-orange to yellow, and the spectrum of the acidified solution became essentially identical to that of the aqueous solution of the untreated fraction. Fractions A and B were extracted from the 0.1 M cyanide solution, and were applied to paper strips for electrophoresis. The color of the applied samples changed from red-orange to yellow when the strips were dampened with acetic acid-cyanide buffer. The color shifts observed upon acidification of the cyanide solutions suggest a conversion of the pigments from a dicyano to a monocyano form, similar to that commonly observed with vitamin B_{12}. Upon electrophoresis in acetic acid-cyanide buffer, the cyanide-treated fractions moved as single zones and had the same mobilities as the corresponding original, untreated fractions.

Fractions A and B showed practically no growth-supporting activity for *L. leichmannii*. With the activity of vitamin B_{12} set equal to 1.0, the activities of equal weights of A and B were 9.9×10^{-3} and 2.4×10^{-2}, respectively.

Separation of pigments by ion-exchange chromatography. Fractions A and B each separated into four radioactive, yellow fractions upon chromatography on carboxymethylcellulose. Upon development of a column with water or 0.001 N acetic acid, fraction A yielded four yellow subfractions. A minor yellow fraction eluted readily with water, a major fraction (A-1) with 0.01 N acetic acid, another major fraction (A-2) with 0.1 N acetic acid, and finally a minor yellow fraction with 1 N acetic acid. Fraction B gave almost the same pattern; the major fractions, B-1 and

B-2, were eluted with 0.01 and 0.1 N acetic acid, in that order. The spectra of aqueous solutions of the main subfractions are shown in Fig. 4 and 5. Each of the subfractions shows absorption maxima in the 300- to 400-mμ region, in the 450- to 500-mμ region, and in the ultraviolet region, similar to those in the spectra of the crude fractions. None of the fractions shows the maximum at 361 mμ characteristic of vitamin B_{12}.

The ion-exchange column fractions were subjected to paper electrophoresis in acetic acid-cyanide buffer. Fractions A-1 and A-2 had the same mobility as did crude fraction A; B-1 and B-2 had the same mobility as did crude fraction B.

Repeated chromatography of the subfractions on carboxymethylcellulose failed to yield single zones, suggesting a slow decomposition of the pigments. Chromatography of small samples under diffuse, red light, however, gave more homogeneous, stable fractions, indicating that the slow decomposition was at least partially caused by light. Investigation of products which have been protected from light is in progress.

Discussion

The electrophoretic mobilities and spectral properties of fractions A and B suggest that these fractions are identical with, or at least very similar to, pigments I and II, respectively, isolated by Helgeland et al. (1961). The formation of our pigment fractions, like that of pigments I and II, requires anaerobic conditions and the inclusion of an energy source in the medium (Scott et al., 1964). The low growth-promoting activities for *L. leichmannii* observed for fractions A and B are also in agreement with the results reported for pigment I (Helgeland et al., 1963).

The presence of Co^{57} label shows that not only fractions A and B, but also the major yellow subfractions of these pigments, are derivatives of vitamin B_{12}. The presence of P^{32} label in the crude fraction suggests that the aminopropanol phosphate skeleton is intact in these derivatives; moreover, all of the fractions show absorption maxima in the ultraviolet region corresponding to the dimethylbenzimidazole nucleotide (Beaven et al., 1950).

In the absorption spectra of fractions A-1, A-2, B-1, and B-2 (Fig. 3 and 4), there are no maxima in the 350- to 370-mμ region, where a maximum characteristic of vitamin B_{12} and many of its analogues and derivatives usually occurs. The maxima in the 310- to 350-mμ and 400- to 500-mμ regions are somewhat similar to those in the spectra of the cobamide coenzymes (Barker et al., 1960; Weissbach, Toohey, and Barker, 1959), some chemically prepared analogues of the coenzymes with cobalt-carbon bonds (Müller and Müller, 1962), and some chemically prepared cobinamide derivatives with inorganic ligands bound to the cobalt (Bernhauer, Renz, and Wagner, 1962). A common property reported for such corrinoids is the sensitivity of the cobalt-ligand bond to cyanide and to light and air. The coenzymes are sensitive to both agents, yielding cyano forms of the corresponding cobamides upon treatment with cyanide, and hydroxo forms upon treatment with light and air. All of the coenzyme analogues with cobalt-carbon bonds are sensitive to one or both treatments, and the cobinamide derivatives are all sensitive to light. Helgeland et al. (1961) suggested as one possibility that their pigment I might have a structure analogous to the coenzymes, and that its increased stability might be attributed to the nature of the ligand bound to the cobalt.

More recently, Hill, Pratt, and Williams (1962) proposed that the yellow pigments isolated by Helgeland et al. (1961) might be substituted on one of the methene bridges. The proposal was based on the properties of a yellow methylcorphyrin prepared by evaporating a solution of hydroxocobalamin and γ-picoline. The residue from the mixture had a spectrum in water or cyanide solution similar to those of the yellow pigments produced by *A. aerogenes*, and showed a similar stability to air and light. Prolonged treatment with light, however, caused a slow decomposition of the synthetic pigment, apparently with complete decomposition of the chromophore.

Compared with the coenzymes and analogous corrinoids with cobalt-carbon bonds, our fractions A and B are stable to cyanide and to light. Although a slow decomposition of the pigments occurs in daylight, electrophoresis of our fractions after prolonged exposure to air and daylight or exposure to 0.1 M cyanide has never revealed the formation of hydroxocobalamin or cyano-cobalamin. It therefore seems less likely that the pigments are simply coordination compounds with different ligands bound to the cobalt, but, more likely, that an alteration of the corrin nucleus has been caused by the bacteria.

Although fractions A and B each give single zones upon electrophoresis, they are heteroge-

nous, each separating into several fractions with cation-exchange chromatography. More complete purification and characterization of these fractions, as well as studies on the effect of light on the pigments, are the objects of further investigation.

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EFFECT OF CORTICOTROPIN ON THE ABSORPTION OF VITAMIN B_{12} IN THE SUBACUTE COMBINED DEGENERATION OF THE SPINAL CORD

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The treatment of subacute combined degeneration of the spinal cord (SCDSC) is based on a pretended etiological unity between the hematologic and neurologic manifestations, according to the old concept of the neuro-anemic syndrome. The first attempt of treatment of that disease was the liver therapy, introduced in 1926 by Minot and Murphy [23], but the neurologic results did not correspond to the success observed in the anemic condition. In 1947, the discovery of the folic acid strengthened the doubts on the etiological unity of the hematologic and neurologic syndromes; actually, the initial findings of Spies et al.[31], later confirmed by several investigators, showed that the folic acid, although improving the blood conditions, had no effect on the neural involvement or even made it worse. The advent of the vitamin B_{12} was received as the solution of the therapeutic problem, for it influenced both the hematologic and neurologic manifestations.

Unfortunately the initial enthusiasm did not last very long and it was found that the neurologic improvement, though generally greater than that yielded by the liver extract alone — and opposite results were reported by Murphy and Howard [25] — was not comparable to the brilliant hematologic effect. In spite of the fact that some authors stress that the deficiency of vitamin B_{12} is a fundamental stone in the building up of SCDSC [24, 27], and although Spillane and Wells [32] have adopted the term "B_{12} neuropathy" suggested by Richmond and Davidson [27], it has been demonstrated that the deficiency of vitamin B_{12} [1, 3, 6, 9, 12, 22, 25, 34, 35] and even achlorhydria [2, 11, 27, 30, 31, 37] are not constant in SCDSC. According to Boudin et al.[3], in SCDSC there is a more complex deficiency than an isolated avitaminosis B_{12}. And even Richmond and Davidson [27], owing to tome significant exceptions to the rule of vitamin B_{12} deficiency, were constrained to formulate the hypothesis that another substance, neuropoetin, besides vitamin B_{12} but closely related to it, is necessary for the integrity of the nervous system.

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On the other hand, the long-term study of the experimental demyelinating encephalomyelitides (Wolf et al., apud Kolb [20]) showed histopathologic features in many ways comparable with those of the so-called primary demyelinations, the poverty of the inflammatory process, the reaction of the astroglia, and in the oldest lesions the relative preservation of the axons and the marked gliosis, deserving particular mention.

Taking into account that such experimental demyelinations have an allergic mechanism, and owing to the good results afforded by ACTH in some demyelinating diseases, we started in 1953 to use it in the treatment of SCDSC [5]. The results were fully analyzed in 1960 [4] and it was evidenced that ACTH alone had an effect similar to liver extract, though inferior to vitamin B_{12}.

Therefore, corticotropin was used on the assumption that the demyelination of SCDSC was due to an auto-immune process, a viewpoint defended by Roger [28] in 1954. Now, however, we ought to accept an additional justification for the use of ACTH in the treatment of SCDSC, owing to the increase of vitamin B_{12} absorption promoted by this hormone, according to the pioneer report of Glass (1955) in a case of sprue [15]. Since then, many other authors [7, 8, 9, 10, 14, 16, 17, 21, 33] have confirmed that finding, regarding both the ACTH and corticosteroids, and either in the malabsorption syndrome or in pernicious anemia. It was demonstrated also that the hypophysectomy and the adrenalectomy cause a deficiency of vitamin B_{12} absorption which can be counteracted, respectively, by ACTH and corticosteroids [36].

The hypothesis that corticotropin would enhance the secretion of intrinsic factor [16] is hardly admissible, because it woud not be in agreement with the results in cases of pernicious anemia with atrophic gastritis [15]. It is more probable that the hormone interferes at the intestinal level, enhancing the absorption of the vitamin regardless of a binding with intrinsic factor to make up the B_{12}-IF complex. Besides this way of action, ACTH mobilizes the organic stores of vitamin B_{12} (Meites et al., apud Glass [15]).

Another mechanism of the ACTH action would be through the increment of the pyridoxine synthesis [18, 26], since the deficiency of vitamin B_6, leading to cortico-adrenal atrophy [36], gives rise to a vicious circle hindering the absorption of vitamin B_{12}. On the other hand, in swines, a B_6-deficient diet can produce demyelination of the peripheral nerves and dorsal funiculi [33].

In this paper the effect of ACTH on vitamin B_{12} absorption in 9 cases of SCDSC is analyzed.

MATERIAL AND METHODS

Material — Nine cases of SCDSC were studied. The diagnosis was based on the following criteria: (a) Characteristic neurologic picture, always represented, in varied degree, by peripheral nerves and dorsal funiculi involvement, and in most cases by a pyramidal syndrome. (b) Gastric anacidity in every case, histamine-fast in all cases but one (case 5); achlorhydria was constitutional in 7 cases and due to partial gastric resection in cases 5 and 8. (c) Changes of the peripheral blood in 6 cases, either in the sense of macrocytic hyperchromic or iron-deficient anemia. (d) Inhibition of maturation at bone-marrow examination in 4 cases.

According to the results of the urinary excretion test of labeled vitamin B_{12}, the diagnosis were: malabsorption syndrome (4 cases), deficiency of intrinsic factor, pernicious-anemia type (2), and undetermined deficient absorption (3).

Methods — Corticotropin was administered by intravenous perfusion in an average dosis of 12.5 to 25.0 mgm./day, diluted in 500 ml. of a 10 percent glucose solution, at a rate or 20 drops/minute. The therapeutic scheme lasted from 19 to 43 days, and the total dosis of ACTH varied from 237 to 505 mgm. (Table 1). The usual care with this kind of treatment was taken.

The absorption of labeled vitamin B_{12} (formerly Co^{60} and later Co^{57}) was studied through the Schilling's urinary excretion test[12]. Excretion levels lower than 10 percent were considered as indicative of deficient absorption. When abnormal results were found, the test was repeated with the association of intrinsic factor *, except, in the last three cases.

The Schilling's test was repeated either immediately after completion of treatment (6 cases) or 28 days (2 cases) and 45 days later (1 case). In cases 3 and 4 the test with vitamin B_{12} plus intrinsic factor was done, respectively, 10 and 5 days after the treatment with ACTH had been started (table 1), thus introducing an error of interpretation concerning the effect of intrinsic factor on the absorption of the vitamin, at least in case 3, where an increased absorption was evidenced.

RESULTS

The results are summarized in tables 1 and 2. Except in cases 6 and 9 an increase in the absorption of vitamin B_{12} was found. The mean difference of the whole group of cases is in the limit of significance.

Case N.º	File Nº	ACTH		Excretion of B_{12} (percent)			Number of days after completion of treatment	Interval between last UET and test after completion of treatment
		Days	Total dosis (mgm)	Before ACTH		After ACTH		
				Single	Plus IF			
1	5108	32	265	5.41	14.03	7.23	45	62
2	5875	20	250	1.61	14.51	3.09	1	26
3	5181	19	237	0.50	8.15	11.53	1	9
4	5210	20	250	6.93	3.60	11.01	1	15
5	5322	19	237	8.65	4.55	15.89	1	37
6	5466	22	275	0.97	3.78	0.63	1	56
7	3385	23	287	8.14	—	11.36	28	59
8	4846	43	505	2.39	—	4.50	28	28
9	5757	20	500	9.18	—	4.70	1	15

Table 1 — Effect of ACTH on vitamin B_{12} absorption in cases of subacute combined degeneration of the spinal cord.

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Case	Diagnosis	Difference of B_{12} excretion after ACTH
1	Deficiency of intrinsic factor	+ 1.82
2	Deficiency of intrinsic factor	+ 1.48
3	Malabsorption	+ 11.03
4	Malabsorption	+ 5.08
5	Malabsorption	+ 7.24
6	Malabsorption	− 0.34
7	Undetermined deficient absorption	+ 3.22
8	Undetermined deficient absorption	+ 2.11
9	Undetermined deficient absorption	− 4.48
Mean difference		+ 3.02 ± 4.45
Significance of the mean		$t = 2.041$
Probability		$0.1 < P < 0.05$

Table 2 — Statistical analysis of the effect of ACTH on radiactive vitamin B_{12} absorption in cases of subacute combined degeneration of the spinal cord.

DISCUSSION

The increase of vitamin B_{12} absorption was observed either in cases of malabsorption syndrome or in cases of lack of intrinsic factor probably due to gastric atrophy. In case 1, with a pernicious anemia pattern of deficient B_{12} absorption, a test of absorption of labeled triolein showed steatorrhea, suggesting that there was also impairment of the intestinal absorption, besides the lack of intrinsic factor. The effect of ACTH was evidenced also in two patients submitted to partial gastric resection (we have no experience in cases of total gastrectomy, a condition in which Gordin[15] found no effect of prednisone on the vitamin B_{12} absorption). In one of these cases (n.º 5) the test with labeled triolein showed a fecal excretion of 31.4 percent, pointing to a marked degree of steatorrhea; so, in this case, the ineffectiveness of the association of intrinsic factor and the deficient fat absorption pointed to an impairment of the absorption of vitamin B_{12} through the intestinal wall.

The failure of ACTH to increase vitamin B_{12} absorption occurred in a case with undetermined deficient absorption (the test B_{12} + IF was not done) and in a case of malabsorption syndrome.

Though the rate of increment in B_{12} absorption after the ACTH series was small in most of cases, the results in cases 3 and 5 must be emphasized, since the degree of urinary excretion raised, respectively, more than 11 and 7 percent. It deserves to be stressed that in these two cases there was a malabsorption syndrome, namely an impairment of the vitamin B_{12} absorption through the ileal mucosa.

In order to study a possible mobilizing effect of ACTH on the radioactive vitamin B_{12} stores, the intervals between the last urinary excretion test and the test after completion of treatment were analyzed (Table 1). It is easily noticed that in the 7 cases with increase of vitamin B_{12} absorption these periods were the most varied, lasting from 9 to 62 days. And also in the 2 cases with decrease of absorption the intervals were very dissimilar, namely 15 and 56 days. So, it seems that this effect of ACTH did not play a significant role in the results.

Likewise, the time elapsed between the completion of treatment and the repetition of the Schilling's test had no apparent relationship with the results.

SUMMARY

The absortion of vitamin B_{12} was studied in 9 cases of subacute combined degeneration of the spinal cord before and after the administration of intravenous ACTH in a therapeutic scheme lasting from 19 to 43 days. In 7 cases an increase of absorption was evidenced. The mean difference of change in the urinary excretion test of radioactive vitamin B_{12} was near the limit of significance.

This finding reinforces the indication of the use of corticotropin in subacute combined degeneration of the spinal cord, since the hormone will act both on the allergic component of the demyelinating process and on the deficiency of vitamin B_{12} absorption. Though the material here analyzed is too small to warrant a definite conclusion, our results suggest that ACTH influences the vitamin B_{12} absorption through the intestinal wall, and not by means of an increase of intrinsic factor secretion or a mobilization of vitamin B_{12} stores.

RESUMO

Efeito da corticotropina sôbre a absorção de vitamina B_{12} na mielose funicular

Foi estudada a absorção de vitamina B_{12} em 9 casos de mielose funicular antes e após a administração de ACTH por via intravenosa em um esquema terapêutico com duração variável entre 19 e 43 dias. Em 7 casos foi verificado aumento da absorção. A diferença média de modificação no teste de excreção urinária de vitamina B_{12} radioativa situou-se próximo do nivel de significância estatística.

Êste resultado reforça a indicação do emprêgo de corticotropina na mielose funicular, pois o hormônio irá atuar tanto sôbre o componente alérgico do processo desmielinizante, quanto sôbre a carência de vitamina B_{12} resultante do déficit de absorção. Embora o material aqui analisado seja muito pequeno para garantir uma conclusão definitiva, nossos resultados demonstram que, provàvelmente, o ACTH age sôbre a absorção da vitamina B_{12} através da mucosa intestinal, e não mediante o aumento de secreção do fator intrinseco ou a mobilização dos depósitos dessa vitamina.

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Serum-Vitamin-B_{12} Concentration in the Elderly

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During the past decade micro-biological assay techniques, using a variety of organisms, have been developed to measure the quantity of vitamin B_{12} in body fluids. Most attention has been directed to the concentration of the vitamin in the serum, where its greatest proportion is normally combined as a labile complex with the α-globulin fraction of the protein. The serum value usually assayed is the amount present following its extraction from the protein complex; this has been termed the total serum-vitamin-B_{12} concentration. Measurements of this concentration in normal people by different investigators have shown considerable variation. The normal mean level, however, appears to be about 400 $\mu\mu$g per ml, with a range extending between 60 and 600 $\mu\mu$g per ml (*Shinton*, 1959).

Mollin and Ross (1952) found a significantly lower mean level in a group of elderly patients over 70 than in healthy hospital staff under 40, the respective means being 358 and 259 $\mu\mu$g per ml. *Boger, Wright, Strickland, Gyle and Ciminera* (1955) found a decrease in serum-vitamin-B_{12} levels with advancing years in two groups of females living in institutions (age range 15–90 years); they found a similar trend in a group of male blood donors (age 20–55 years), but no age difference in another group of males living in an institution (age range 14–86 years). *Chow, Wood, Horonick and Okuda* (1956) investigating similar groups of people drawn from the general population and from residents in institutions, found a lowering of the mean serum-B_{12} level in the elderly. They later

confirmed this in an extended study with a larger number of subjects (*Gaffney, Horevich, Okuda, Meier, Choie and Stock, 1957*). In the latter study no "substantial difference" in levels was found between males and females. The validity of this general trend towards lower serum-B_{12} levels with age was challenged by *Killander* (1957) and by *Kristensen* (1958). Both of these authors found no significant difference with age, but in their studies the elderly "normals" were hospital patients. These findings were later supported by *Droller and Dossett* (1959) whose subjects were also hospital patients. They noted, however, a significantly lower mean level in a group of demented old people. This created further interest in the significance of serum-B_{12} levels in the elderly and of the possible importance of the lower values sometimes found in them.

It has recently again been reported (*Holmes*, 1956; *Weiner and Hope*, 1959, and *Fraser*, 1960) that deficiency of vitamin B_{12} may lead to psychiatric disturbances. *Holmes* (1956) described fourteen cases presenting 'cerebral manifestations' of vitamin-B_{12} deficiency. In thirteen of his fourteen cases there was some degree of megaloblastic change in the marrow, while all cases had a histamine-fast achlorhydria. In the one case with a normal bone marrow the diagnosis was based on the achlorhydria and a satisfactory response to treatment, which was originally largely speculative, with vitamin B_{12}. When this study was carried out there were no facilities for assaying serum-B_{12} levels. The author emphasized that such an assay would be a crucial investigation and quoted an additional case with dementia, ataxic paraplegia, normal blood values and bone marrow, but with a serum-B_{12} level of 90 $\mu\mu$g per ml. This case responded to large doses of the vitamin. Two of the cases came to post mortem. In both there was typical microscopical appearance of "status spongiosus" in the spinal cord, while areas of degeneration were scattered throughout the cerebral white matter. The staining reactions of the cerebral lesions were in all respects similar to those in the spinal cord. *Holmes* was thus able to add evidence to *Adams and Kubik's* (1944) finding that the cerebral and cord lesions are alike.

Holmes describes the clinical features of these cerebral manifestations as falling into two groups, ophthalmological and mental. The former consist mainly of dimness of vision due to optic atrophy; the latter are extremely variable and include "mild disorders of mood, mental slowness, memory defect which may be gross, con-

fusion, severe agitation and depression, delusions and paranoid behaviour, visual and auditory hallucinations, urinary and faecal incontinence without overt spinal lesions, dysphasia, violent maniacal behaviour and epilepsy". Such a catalogue of mental disturbances might well represent the symptoms of senile dementia. This resemblance has led to speculation on a possible role of vitamin-B_{12} deficiency in the production of senile dementia. *Droller and Dossett* (1959) studied the serum-B_{12} levels of four groups of elderly patients whom they termed "normal, demented, confused and pernicious anaemia". They reported significantly lower serum-vitamin-B_{12} levels in senile demented patients than in normals of the same age. Confused patients had less marked deficiency, but showed a large proportion of "intermediate" values (110–180 $\mu\mu$g per ml). These low and "intermediate" values were not obtained in normal old people.

Because of the lack of conformity in the reported results of the relation of serum-vitamin-B_{12} concentration and age, we measured the levels in apparently healthy subjects of all age groups. We then compared these findings with those found in known cases of pernicious anaemia and subacute combined degeneration of the spinal cord and in elderly patients of a geriatric unit. We designed the investigation of the elderly patients to reveal differences in senile demented and confused patients. There is difficulty in distinguishing between confusion and dementia, but in our view 'demented' implies a loss of orientation in time and space which is persistent and not secondary to physical disorders, such as respiratory, gastrointestinal or cardiac disease. This loss of orientation may be accompanied by agitation, aggressiveness, depression, delusions or hallucinations. Mental confusion, on the other hand, we regard as an episode of temporary dementia. Repeated mild attacks of mental confusion may herald the onset of a permanent dementia, thus prolonged observation may be necessary to establish the diagnosis. For this reason we did not attempt to separate dementia from confusion, and we grouped our cases as normal, geriatric and demented.

Materials and Methods

Total serum-vitamin-B_{12} concentrations were estimated on four groups of subjects, over the age of 60 years:

Group A. Normal population. 30 males and 26 females who were ambulant, living in their own homes and had not attended a hospital either as an out or in-patient for

at least ten years. Those with haemoglobin levels below 11.5 g per 100 ml. were excluded. These subjects were all volunteers, being either visitors to patients in hospital or members of Old Peoples' Community Centres.

Group B. Geriatric patients. 22 patients in a geriatric ward of a general hospital who had been admitted for a variety of reasons. Cases of congestive failure, severe acute or chronic respiratory disease and neoplasia were excluded. Cases of recent cerebrovascular episodes were also excluded, but cases whose cerebral thrombosis had occurred at least ten weeks before the specimen was examined, were included. None of the cases showed any evidence of dementia or mental confusion.

Group C. Demented patients. 20 patients in the same geriatric unit, who were persistently confused due to senile dementia.

Group D. Pernicious anaemia, and sub-acute combined degeneration patients. 24 cases in relapse who had macrocytic anaemia with megaloblastic bone marrow, histamine-fast achlorhydria followed by response to vitamin-B_{12} therapy.

The serum-vitamin-B_{12} concentration was estimated by the method of *Ross* (*Hutner, Bach and Ross*, 1956) using Euglena gracilis Z strain. All sera were assayed on at least two separate occasions, the recorded result being the mean of these readings.

Results

Variation of Serum Concentration with Age in Normal Population

The data obtained from the elderly normal subjects (Group A) has been combined with those obtained from a series of normal people (aged 15–60 years), previously studied by one of us (*Shinton*, 1959) in order to relate changes with age (table I). It can be seen

Table I

Serum-Vitamin-B_{12} Concentration in Various Age Groups

Age group years	No. of subjects	Serum-vitamin-B_{12} Concentration ($\mu\mu$g/ml) Mean	S.D.
15–19	20	359	78
20–29	27	366	119
30–39	20	409	86
40–49	30	388	100
50–59	23	427	174
60–69	25	267	101
70–79	26	308	139
80–89	5	202	76
Under 60	120	390	119
Over 60	56	280	121

that, apart from the group of subjects over 80 years of age, there is no great discrepancy in the standard deviation of the mean serum-B_{12} levels. These mean levels show a slight upward trend before the age of 60 years, after which there is a striking fall (fig. 1). The correlation coefficient for the rise is just significant ($r = -0.206$;

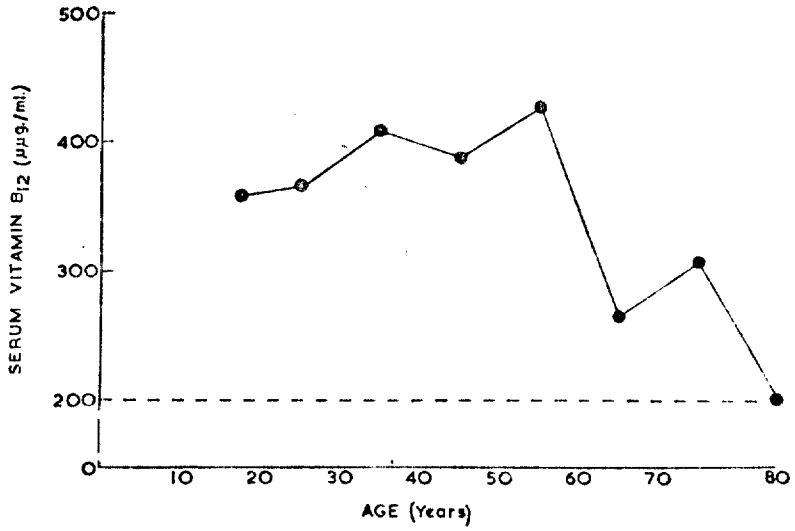

Fig. 1

n = 120; P = <0.05). The difference between the mean levels before and after the age of 60 years is highly significant (t = 5.5; df = 176; P = <0.001). The correlation coefficient of age with B_{12} level in those over 60 years is not significant (r = 0.151; n = 56; P = >0.1). The difference in the levels between normal males and females seen in fig. 2 is not significant, the mean levels being male, 299 $\mu\mu$g per ml. and female 259 $\mu\mu$g per ml. (t = 1.27; df = 45; P = >0.1).

Table II

Age and Serum-Vitamin-B_{12} Concentration in Four Groups of Elderly Subjects

	Group A Normals	Group B Geriatric	Group C Demented	Group D P.A. and S-A.C.D.
Number of subjects	56	22	20	24
Age – Mean	71.73	74.00	81.60	72.08
S.D.	5.67	7.30	6.18	5.76
B_{12} level – Mean	280	300	320	35
S.D.	121	208	136	23

Variations between Elderly Normal Subjects and Geriatric and Demented Patients

The complete data of the serum-B_{12} levels found in the elderly subjects from all groups studied are shown diagramatically in fig. 2

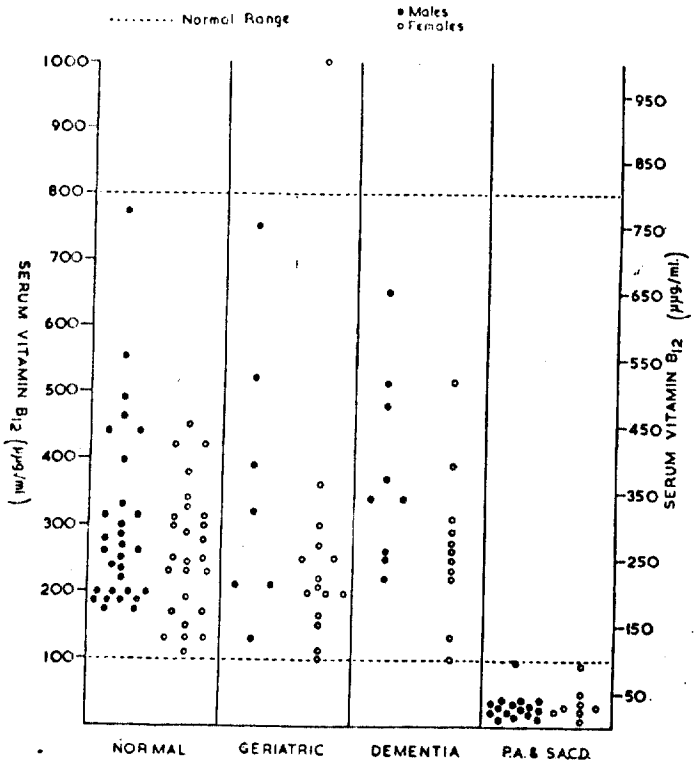

Fig. 2

and summarized in table II. There is little difference between the mean serum levels in the normal, geriatric or demented groups and the correlation coefficient of level with age is not significant. In each of the three groups there is a small number of subjects with levels between 100 and 200 $\mu\mu$g per ml. but there is no evidence to suggest that these lower levels are found more frequently in the demented group. One geriatric patient had a serum-B_{12} concentration of 1000 $\mu\mu$g per ml. This was a woman of 62 years who had had two cerebral thromboses, two years and one year before admission. She had marked Parkinsonian signs with some rigidity of all limbs and severe hypertension (B.P. 260/150). In October 1957 and April 1958 she had courses of intramuscular vitamin B_{12}, 500 μg daily for seven days. The serum-B_{12} assay was performed on the 3rd July, 1958 ten weeks after the second course. At the time of the

assay there was no clinical or haematological evidence of hepatic or myeloproliferative disorder. The assay was repeated four weeks later when the level found was 620 $\mu\mu$g per ml. The patient died on the 29th August, 1958 from a coronary thrombosis, but unfortunately permission for post mortem examination was not granted.

In the two cases of the demented group where the levels were below 200 $\mu\mu$g per ml. the haematological values were normal and there was no clinical evidence of subacute combined degeneration of the cord. Gastric analysis was not performed in either case.

Discussion

The fall noted in the serum-vitamin-B_{12} concentration with age is in agreement with the work of some earlier authors (*Mollin and Ross*, 1954; *Boger* et al., 1955; *Chow* et al., 1956, and *Gaffney* et al., 1957). One point of difference, however, is the sharp fall in the present series at the seventh decade, whereas *Boger* et al. (1955) and *Gaffney* et al. (1957) found a steady decrease from the second decade onwards. To exclude variation in assay techniques in our series the samples from the various age groups were mixed, and some from each group assayed in each batch. There was, however, a tendency for some decades to be represented by a particular group of the population, for example the majority of the subjects of the second decade were medical students and of the fourth blood donors. Similar groups were chosen by *Boger* et al. (1954) with different results, but this could be due to an error in sampling as the two investigations were carried out in distinct geographical areas. Such an error may also explain the absence of any decline in level with age in the series reported by *Killander* (1957), *Kristensen* (1958) and *Droller and Dossett* (1959). These authors based their findings on sera from hospital patients in whom there may have been a proportion of subjects with abnormally high levels.

The difficulties entailed in sampling for the determination of normal serum levels have been commented on by a recent W.H.O. study group on Immunological and Haematological Surveys (1959). While they considered random sampling from all possible localities to be the ideal method this is usually impracticable. A method of avoiding these possible errors was suggested by *Pryce* (1960), who used random samples from all sources, and applied an arithmetical

convention to the results. This author considered that taking samples from selected groups, such as those considered to be "normal", prejudices the results. This criticism can clearly be directed at our study. In spite of this we have found a statistically significant difference between serum-B_{12} levels in young and old healthy subjects. Whether this can be accepted as a normal physiological state or implies a pathological one in the elderly, requires further investigation. Such factors as alteration in diet, impairment of intestinal absorption and change in the plasma protein complex to which vitamin B_{12} is combined, would have to be considered.

In the second part of our study we did not find any significant difference in the serum-B_{12} levels of elderly subjects whether normal, geriatric patients or demented. We did find a small number of cases with levels between 110-180 $\mu\mu$g per ml. corresponding to the 'intermediate range' of *Droller and Dossett*. These cases occurred in all our groups of elderly people in approximately similar proportions in the normal and geriatric groups, but in smaller proportion in the demented group. Our evidence suggests that serum-B_{12} levels in this range do not give rise to clinical syndromes of deficiency of the vitamin and their occurrence has no proven pathological significance. It follows that the administration of the vitamin to patients with levels in this range for mental symptoms has no rational basis.

Authentic cases of psychiatric disorders due to vitamin-B_{12} deficiency have been described (*Holmes*, 1956; *Weiner and Hope*, 1959, and *Fraser*, 1960). All of these had a histamine-fast achlorhydria, all but one had megaloblastic erythropoiesis, and in the two cases where assays were available, serum-B_{12} levels less than 100$\mu\mu$g per ml. Such cases appear to be rare. In our opinion, cases of senile dementia, in whom the serum-B_{12} concentration lies between 110 and 180 $\mu\mu$g per ml., do not fall into this category. Unfortunately in our two demented cases with a B_{12} level in this range, the gastric juice and bone marrow were not examined. Both patients, however, had normal haematological findings. Clinically, the features of both senile dementia and cerebral disturbances due to B_{12} deficiency are protean, and it is impossible to differentiate the one from the other without laboratory assistance. The most satisfactory differential test is a serum-B_{12} assay and levels less than 100 $\mu\mu$g per ml. should be regarded as probably indicating deficiency of the vitamin.

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Summary

Serum-vitamin-B_{12} assays were carried out in ninety-eight elderly subjects.

The subjects were divided into three groups, normal subjects, geriatric patients and demented.

Comparison of the results obtained with those of an earlier study by one of us (*N. K. S.*) on younger persons, revealed a sharp fall in mean serum-B_{12} level at the seventh decade. The implications of this are discussed.

No statistically significant difference was found between the mean levels noted in the three groups of elderly subjects which suggests that vitamin-B_{12} deficiency is not a factor in the production of senile dementia.

Zusammenfassung

Vitamin-B_{12}-Bestimmungen im Serum wurden bei 98 betagten Personen vorgenommen.

Die Personen ließen sich in drei Gruppen unterteilen: normale, geriatrische und senil-demente.

Im Vergleich zu einer früheren Untersuchung durch einen der Autoren (*N. K. S.*) bei jüngern Individuen konnte ein brüskes Absinken des mittleren B_{12}-Gehaltes im Serum im siebenten Dezennium festgestellt werden. Die damit einhergehenden Auswirkungen werden besprochen.

Ein statistisch signifikanter Unterschied im B_{12}-Gehalt des Serums bei den drei Gruppen betagter Personen konnte nicht festgestellt werden. Somit dürfte dem Mangel an Vitamin-B_{12} für die Entwicklung einer senilen Demenz keine Bedeutung zukommen.

Résumé

Des essais ont été effectués pour apprécier le taux sérique de la vitamine B_{12} chez 98 sujets âgés divisés en 3 groupes: normaux, malades âgés et déments.

La comparaison de ces résultats avec ceux obtenus lors d'une étude antérieure, faite par l'un des auteurs, sur des sujets plus jeunes, a révélé une brusque baisse du taux de la vitamine B_{12} dans le sérum, à la 7e décade. Cette constatation est mise en discussion.

Statistiquement on n'a pas trouvé de différence significative entre les taux moyens des 3 groupes de sujets âgés ce qui fait penser que le déficit en vitamine B_{12} n'est pas un facteur actif dans la démence sénile.

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Radioassay for serum vitamin B_{12} with the use of saliva as the vitamin B_{12} binder

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A rapid, easy radioassay for serum vitamin B_{12} is described. Saliva, a readily available, rich source of vitamin B_{12}-binding protein, is used. The assay is reproducible, correlates satisfactorily with microbiological assay results, and clearly differentiates between B_{12}-deficient, normal, and high-B_{12} sera. Poor exclusion by some hemoglobin-coated charcoals of saliva-bound B_{12} in the absence of serum resulted in falsely low results. This problem can be corrected by acidification and redrying of the charcoal powder prior to coating or circumvented by additional coating of the hemoglobin-coated charcoal with vitamin B_{12}-saturated serum.

In view of the various technical problems of microbiological assay, several radioassay procedures for vitamin B_{12} (B_{12}) have been described, with the use of intrinsic factor[1-5] or serum[6-10] for binding the vitamin. These assays employ various means for subsequent separation of free from bound B_{12}. The coated charcoal adsorption method[3-5, 10] is the most rapidly and easily performed but occasionally has been found to give falsely low and, in some B_{12}-deficient sera, negative results.[4, 5]

This report presents a modified coated-charcoal radioassay, with the use of saliva, a rich source of relatively stable and homogenous B_{12}-binding protein.[11, 12]

Materials and methods

Parotid saliva, collected with a cap over the parotid duct opening,[13] and mixed saliva from normal subjects was spun 15 minutes at 3,000 r.p.m. to remove debris. B_{12}-binding capacity was determined with coated charcoal.[14] The saliva was diluted with saline-phosphate buffer, pH 7.2, so that 0.1 ml. bound 300 to 400 pg. of Co^{57} B_{12}. Maximal binding capacity

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Table I. Protocol for serum B_{12} assay

	0.9% saline (ml.)	*Serum (ml.)*	*1N HCl* (ml.)*	*Co^{57} B_{12}† (ml.)*	*Saliva*	*Saline-PO_4 buffer (pH 7.2)‡*
Unknown serum	1.0	0.5	0.5	0.5	§	—
Serum supernate control	1.0	0.5	0.5	0.5	—	§
Saliva standard	1.5	—	0.5	0.5	§	—
Saliva supernate control	1.5	—	0.5	0.5	—	§

*Mix 10 seconds in a Vortex mixer; cap with cotton wool; place in boiling water bath for 30 minutes. Cool in tap water for 10 minutes.

†Mix 10 seconds in a Vortex mixer.

‡Mix 10 seconds in a Vortex mixer, incubate 30 minutes at 23° C.; add 2 ml. of coated charcoal to each sample; mix 10 seconds in a Vortex mixer twice. Spin 15 minutes at 3,000 r.p.m., and count supernate radioactivity.

§That volume of diluted saliva binding 300 to 400 pg. of Co^{57} B_{12} is used, usually 0.2 to 0.3 ml.; an equivalent volume of buffered saline is used in the other samples.

was established by incubating 0.1 ml. of diluted saliva with increasing amounts of Co^{57} B_{12}. A B_{12}-binding curve was determined by incubating increasing amounts of diluted saliva with 500 pg. of Co^{57} B_{12}. In each experiment, unbound Co^{57} B_{12} was removed with hemoglobin-coated charcoal. Aliquots of diluted saliva were stored at -20° C. as long as 4 months.

Sera from healthy volunteers, patients with pernicious anemia (confirmed by bone marrow examination and Schilling tests), and untreated patients with chronic myelogenous leukemia (diagnosis based on National Cancer Institute criteria[15]) were stored at -20° C.

Hemoglobin-coated charcoal was prepared as described by Lau and associates.[3] In later experiments, charcoal was either: (1) further coated with 4 ml. of pooled serum (or plasma), previously incubated for 30 minutes with excess B_{12}, to 100 ml. of hemoglobin-coated charcoal, or (2) preacidified by treating it with 1N HCl for 2 hours, repeatedly washed with deionized water, readjusting pH to 7 with 1N NaOH and rewashing with water, and dried at 100° C. for 24 hours in a drying oven. The activated charcoals tried were Norit A, untreated powder* (Sigma); Norit SG Extra† ; neutral Norit A, pharmaceutical grade‡ (Amend); and charcoal No. 4394, NF§ (Mallinckrodt). Unless otherwise specified, only the first 2 were used in our studies. Both are from a peat source and have a pH of 9.8 and 7.8, respectively, in water.

Co^{57} B_{12}, specific activity 11 to 16 μCi per microgram,‖ was diluted to 1,000 pg. per milliliter with saline-phosphate buffer, pH 7.2.

The assay protocol, shown in Table I, was modified from the method of Lau and associates.[3] All samples were assayed in duplicate, and radioactivity was counted in a well-type scintillation counter. In added experiments, 0.5 ml. of serum was incubated with 2 ml. of 0.2M acetate buffer, pH 4.5, with autoclaving for 15 minutes at 120° C., cooling, centrifuging, and addition of 500 pg. of Co^{57} B_{12} and 0.2 ml. of NaCN (10 μg per milliliter) to the resulting supernate before adding saliva and charcoal. Controls listed in Table I were treated similarly.

Assay results were calculated by the equation of Lau and associates[3] from counts-per-minute values:

$$\text{picograms of } B_{12} \text{ per milliliter of serum} = 2 \times 500 \left(\frac{\text{Saliva standard} - \text{saliva supernate control}}{\text{Unknown serum} - \text{serum supernate control}} - 1 \right)$$

*Sigma Chemical Co., St. Louis, Mo.
†American Norit Co., Jacksonville, Fla.
‡Amend Drug & Chemical Co., New York, N. Y.
§Mallinckrodt Co., St. Louis, Mo.
‖Abbott Laboratories, North Chicago, Ill.

Standard curves were constructed by assaying, serially, 100 to 1,000 pg. of added crystalline B_{12} with saline or 0.5 ml. of serum.

Chronic myelogenous leukemia sera were assayed in dilution by using 0.1, 0.2, 0.3, 0.4, and 0.5 ml. of serum.

Effect of the charcoals on B_{12} binding by saliva, neutralized depepsinized[16] normal human gastric juice, and serum was tested by incubating the specimens, in combination and separately, with excess Co^{57} B_{12} and adding 2 ml. of the different hemoglobin-coated charcoal suspensions. After mixing and centrifuging, the supernate (i.e., bound) radioactivity was determined.

Results

Mixed saliva bound more added B_{12} than did parotid saliva from the same subject (60, 55, and 39 ng. per milliliter compared with 16, 18, and 9 ng. per milliliter, respectively). The mean B_{12}-binding capacity of 7 mixed samples was

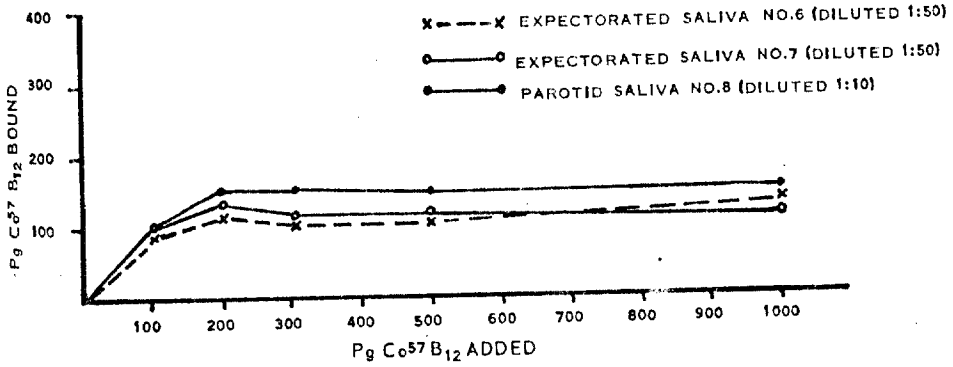

Fig. 1. Co^{57} B_{12} binding by 0.1 ml. of diluted saliva in the presence of excess Co^{57} B_{12}.

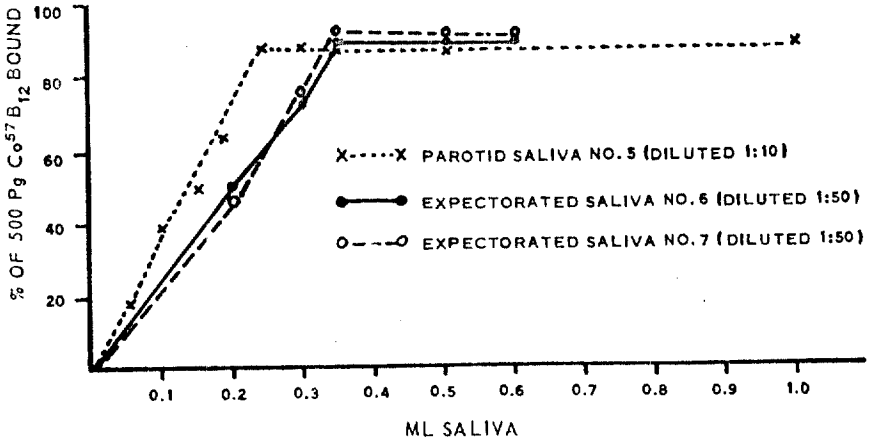

Fig. 2. Saliva binding of 500 pg. of Co^{57} B_{12}.

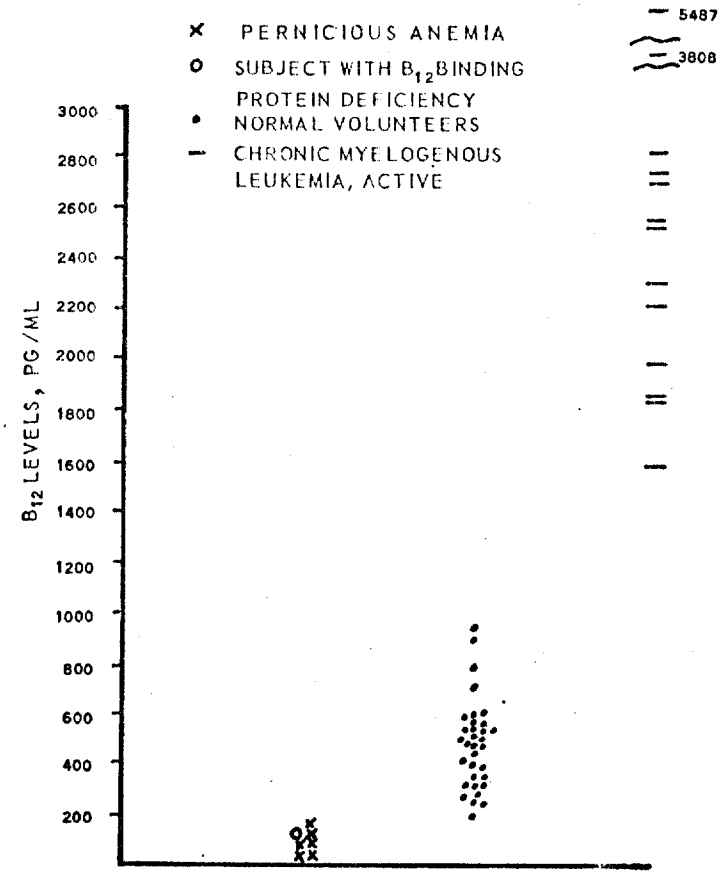

Fig. 3. Serum vitamin B_{12} assay results.

40 ng. per milliliter (range, 23 to 66 ng. per milliliter). The binding capacity of saliva obtained at different times from the same subject varied, but each specimen gave consistent results. Two specimens, tested after 3 months of use, retained 74 and 100 per cent binding capacity, while 2 others retained 74 and 89 per cent after 6 months. Thus, a standardized saliva could be used for 2 months before volume increase was required to compensate for loss of binding.

A fixed maximal binding of B_{12} was reached with parotid and mixed saliva, despite great excess of Co^{57} B_{12} (Fig. 1). Appropriate dilutions of parotid and mixed salivas bound up to 85 to 90 per cent of 500 pg. of Co^{57} B_{12} (Fig. 2). From this curve, the amount binding 70 to 75 per cent was found for subsequent use in assay. Adequate binding of B_{12} occurred at 30 minutes, with no increase resulting even after 4 hours of incubation.

Assay results are presented in Fig. 3. B_{12} levels of 32 healthy volunteers ranged from 205 to 937 pg. per milliliter (mean, 471 pg. per milliliter ± 174). Six subjects with pernicious anemia had levels of 17 to 165 pg. per milliliter.

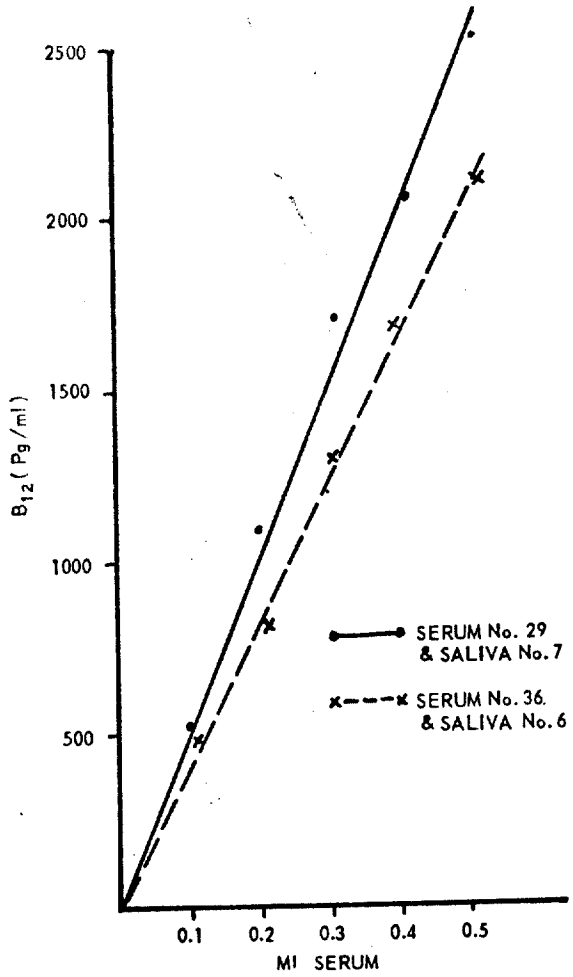

Fig. 4. B_{12} assay of increasing dilutions of chronic myelogenous leukemia sera, with the use of different saliva specimens.

No negative results were seen. Thirteen patients with untreated chronic myelogenous leukemia had levels of 1,582 to 5,487 pg. per milliliter. Serum from a subject with B_{12}-binding protein deficiency[17] contained 101 pg. per milliliter, compared with 36 pg. per milliliter by the method of Lau and associates.*[3] In 600 sera subsequently assayed in our laboratory, clear differentiation between deficient, normal, and high-B_{12} sera has been maintained. Inclusion of supernate controls is mandatory with each sample, since these can vary significantly from serum to serum.

Variance of levels of 44 sera, reassayed one day to three months later with

*Performed in the laboratory of Dr. V. Herbert, The Mt. Sinai School of Medicine, N. Y., who kindly sent us the serum samples.

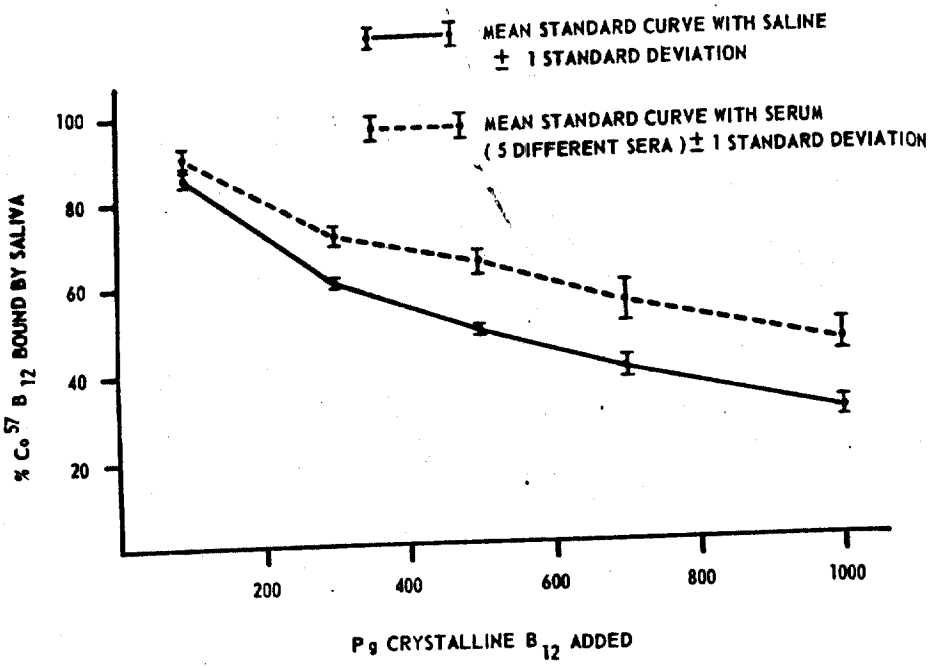

Fig. 5. Standard curves constructed with saline and with serum.

different saliva specimens, averaged 11 per cent from the mean value, the same as noted in duplicates within the same assay. Increasing dilutions of sera from 2 patients with chronic myelogenous leukemia gave B_{12} levels showing consistent recovery of endogenous B_{12} (Fig. 4).

The assay system with the use of saliva, like intrinsic factor[3] but unlike serum,[8, 9, 18] obeyed the principle of isotope dilution[19] perfectly in saline. However, when the dilution curve was constructed in the presence of serum, a consistent overestimation of 500 pg. of Co^{57} B_{12} as 850 pg. ± 100 occurred. When our results were calculated from a standard curve with the use of saline (Fig. 5), low B_{12} values were obtained. Acceptable values resulted when based on a standard curve in serum. However, variation by as much as 100 pg. per milliliter occurred, depending on the particular serum used in constructing the curve. Although identical standard curves were obtained with the same serum, different sera gave slightly different curves (Fig. 5). Therefore, standard curves were replaced by the equation of Lau and associates[3] in our calculations.

Results of our assay of 20 sera compared favorably with the results of the *Euglena gracilis* assay*[20] (correlation coefficient, 0.9745), the lone discrepany being in a serum which may have contained antibiotics. Extraction of B_{12} with cyanide instead of acid-boiling did not improve results. On the contrary, several falsely low levels were noted.

Low B_{12} levels, with occasional negative values for B_{12}-deficient sera, oc-

*Performed by Drs. M. E. Haggard and J. B. Alperin, University of Texas Medical Branch, Galveston, Texas.

Table II. Saliva and gastric juice binding of vitamin B_{12} in the absence and presence of serum, with the use of various kinds of Norit A charcoal.

	B_{12}-binding (pg.)	
	Sigma Charcoal	*Amend Charcoal*
Saliva No. 14	5,421	1,224
Saliva No. 14 + Serum No. 574	5,654	5,688
Gastric juice No. 3	3,126	1,983
Gastric juice No. 3 + Serum No. 574	3,679	3,609
Serum No. 574	554	596

curred when Amend or Mallinckrodt charcoals were used in our assay. In every case, the lower B_{12} levels were due to low saliva standard values, the supernate control and test serum values remaining the same. The 2 charcoals excluded less saliva-bound (or gastric juice–bound) Co^{57} B_{12}, resulting in a "low B_{12}-binding capacity." This was true with more than half of the saliva specimens tested. When the experiment in Fig. 2 was repeated with Mallinckrodt charcoal, no more than 57 per cent binding occurred regardless of the amount of saliva added. Adding serum to a saliva or gastric juice specimen raised the total binding capacity to that seen with Sigma charcoal (Table II).

Subsequently, low salivary B_{12} binding (and negative serum B_{12} levels in assay) was also seen with a batch of Sigma charcoal which had been standing on the shelf in powder form for 4 months. This was completely corrected when the charcoal powder was treated with acid and redried prior to coating. Alternatively, by further coating the hemoglobin-coated charcoals with B_{12}-saturated serum prior to use, the artifactually low salivary B_{12}-binding was corrected, and satisfactory B_{12} assay values resulted with any of the charcoals.

Discussion

Various B_{12} binders have been used in B_{12} radioassay. Intrinsic factor has the disadvantage of relative instability and increasing B_{12}-binding capacity when exposed to excess B_{12}[9] and is not readily available. Serum contains several types of B_{12}-binding protein[21, 22] and binds excess B_{12} nonspecificically.[9, 18] High Transcobalamin I serum also exhibits nonspecific binding, though to a lesser degree,[9, 18] is not always easily available in large quantities, and contains less than a tenth of the B_{12}-binding capacity of saliva.

Salivary B_{12} binder is easily obtained (mixed saliva is a satisfactory source), requires little manipulation, and can be stored at –20° C. for 2 months with little loss of potency, even when thawed and refrozen. Saliva contains predominantly one B_{12} binder, [12, 23] which resembles serum transcobalamin I and has an estimated molecular weight of 60,000.[23] The plateau of maximal binding shown in Fig. 1 further suggests absence of nonspecific binders.

Good separation of B_{12}-deficient ($<$ 150 pg. per milliliter) and high B_{12} ($>$ 1,000 pg. per milliliter) from normal sera was obtained with our assay. Levels between 150 and 200 pg. per milliliter were considered indeterminate.

Results correlated satisfactorily with microbiological assay, and reproducibility approximated that of other radioassays.[4, 5] Consistent levels over wide ranges of B_{12} content were evident from data in Fig. 4, in contrast with results by other assays.[4, 5]

In a system which behaves differently in saline than in serum, a standard curve with saline cannot be used. The saliva radioassay, by obeying the principle of isotope dilution in saline only, is such a system. Constructing a curve with serum is necessary but results in variations of the B_{12} level by 100 pg. per milliliter depending on the serum used for the standard curve. To maintain absolute accuracy in the saliva assay, and probably in all B_{12} radioassays to data, a separate curve with the use of each serum to be assayed would have to be constructed. Simple use of the equation of Lau and associates,[3] derived from Rittenberg and Foster,[24] resulted in satisfactory accuracy in our assay, as judged by comparison with calculations from various standard curves in serum and with microbiological assay results.

In previous reports,[2, 4, 5, 9, 25] serum interacted occasionally with intrinsic factor in an undefined way, increasing B_{12} binding by the latter. This was subsequently demonstrated to be due to coating of the charcoal by the added serum.[14] Similarly, the low B_{12} binding by saliva in our assay in the presence of some hemoglobin-coated charcoals probably was due to decreased exclusion of saliva-bound B_{12}. Thus, falsely low or even negative results were seen, due to low saliva standard (which contains no serum) values compared with the test serum values. That this occurred with some but not all charcoals reflects the fact that all activated charcoals are not identical.[26, 27] Thus, Norit SG Extra and several batches of Sigma charcoal gave satisfactory results. Although initial pH of the charcoal bore no relation to its suitability, deterioration of one batch was corrected by treating with acid, readjusting to pH 7, and drying, possibly thereby removing accumulated impurities or in some fashion reactivating the charcoal. The suitability of the charcoal may be checked periodically by the experiment in Table II. It was found that the entire problem could be circumvented by additional coating with serum of the hemoglobin-coated charcoal, prior to use in assay, in order to satisfactorily exclude salivary binders. Other coating materials may be used also, if they are in accordance with charcoal assay principles[28] and can be shown to exclude satisfactorily the B_{12} bound by saliva.

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THE VITAMIN B_{12}-BINDING PROTEINS OF SALIVA AND TEARS AND THEIR RELATIONSHIP TO OTHER VITAMIN B_{12} BINDERS

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SUMMARY

A study of the vitamin B_{12} binder in saliva was prompted by different reported molecular weights, some suggesting it was unique among the otherwise identical group of vitamin B_{12} binders called R binders. Tears, extremely rich in this protein, were also examined.

Gel filtration and sucrose density gradient ultracentrifugation showed saliva binder to be identical in size with other R binders, serum third binder and transcobalamin I. The discrepancy with previous reports is unexplained. It is apparent that vitamin B_{12} binders are anomalous proteins, and past estimates of molecular weight may be incorrect.

Saliva binder resembled leucocyte binder and serum third binder, but not transcobalamin I, in electrophoretic mobility. However, some heterogeneity was obvious, even among different specimens from the same subject. Tear binder was intermediate between R binder and transcobalamin I on electrophoresis.

These results support identity of all the R binders except for some heterogeneity, probably acquired, in isoelectric points. It also appears that third binder is the R binder in serum.

INTRODUCTION

A single type of vitamin B_{12}-binding protein (as determined by immunologic and chromatographic behaviour and molecular sizing) appears in most of the body fluids of man and in leucocytes and erythrocytes[1,2], and is called "R binder"[1,3]. The sole exception in the similarity of all R binders to each other has been saliva binder, for which different molecular weights have been reported[4-6].

Although R binder is present in serum[1], it is uncertain which serum binder corresponds to it due to the variability of separation techniques. The non-identity of R binder with transcobalamin II has been summarized[7]. Transcobalamin I differs from R binder electrophoretically[8,9], and serum R binder does not carry endogenous vitamin B_{12} (ref. 1). The recently described third serum binder[10-13], however, resembles R binder in all tested characteristics[10,13].

The present study was undertaken to further examine saliva binder and its possible resemblance to third binder of serum, and to determine whether it indeed differs in molecular size from other R binders. Tear vitamin B_{12}-binding protein was similarly studied. Tears have exceptionally high vitamin B_{12}-binding capacity, yet the binder has not been characterized apart from determination of electrophoretic mobility at pH 8.6 (ref. 14).

MATERIALS AND METHODS

Saliva specimens were collected by unstimulated spitting from four fasting, normal subjects and one fasting subject with pernicious anaemia in remission. Spontaneous tears were collected by eye-dropper from two healthy children and pooled. Samples were centrifuged to remove debris, but were otherwise untreated. Testing was done immediately or after storage at −20 °C, with no difference in results. *Polycythaemia vera* serum was used as the source of third binder.

Sephadex G-200 gel (Pharmacia Fine Chemicals, Uppsala, Sweden) filtration was done at room temperature in 0.9 cm × 60 cm columns, using 1 M NaCl–0.1 M Tris buffer (pH 8.5) containing 0.02% NaN_3. Samples of 0.1–0.3 ml were incubated with excess [^{57}Co]vitamin B_{12} (The Radiochemical Centre, Amersham) specific activity of 15.2 μCi/μg, for 30 min at room temperature prior to application. Aliquots of 10 drops were collected by gravity. Radioactivity was counted in a well-type scintillation counter. Sephadex G-75 gel filtration was similarly performed, using the above buffer or 0.1 M sodium phosphate buffer (pH 6.3). Both gel columns had been calibrated with high-molecular weight Blue Dextran, [^{57}Co]vitamin B_{12}-labelled leucocyte extract[9], and various [^{57}Co]vitamin B_{12}-labelled human sera. Protein concentration was determined at 250 nm during flow.

Three of the saliva specimens were filtered through Sephadex G-200 or G-75 without added [^{57}Co]vitamin B_{12}. Filtrate tubes were then each incubated with 1 ng [^{57}Co]vitamin B_{12} for 30 min at room temperature, and the unbound [^{57}Co]vitamin B_{12} was removed with haemoglobin- and plasma-coated charcoal[15] prior to counting.

Linear sucrose density gradient ultracentrifugation of diluted saliva and serum, labelled with [^{57}Co]vitamin B_{12}, specific activity of 118 μCi/μg, was done as described by Martin and Ames[16], spinning 17.5 h at 38 000 rev./min with a 3 × 6.5 swing-out rotor in a Superspeed 65 centrifuge (Measuring and Scientific Equipment, London) at 7 °C. Transferrin, IgG and human serum albumin, all labelled with ^{125}I, were used as internal standards.

Cellulose acetate electrophoresis was done at pH 8.6 on specimens previously incubated with [^{57}Co]vitamin B_{12}, specific activity of 118 μCi/μg. Serum labelled with ^{59}Fe was used for comparison. The unstained strips were incubated for 3 weeks with no-grid X-ray film, and stained subsequently for comparison with the radioautographs.

Pevikon (Shandon Scientific, London, U.K.) block electrophoresis at pH 4.5 was done on three separate occasions as described elsewhere[9].

RESULTS

Five saliva samples from three subjects had identical elution patterns on

serum third binder and leucocyte binder, but not transcobalamin I (Fig. 2). Once in three electrophoreses, both salivas had a small cathodal "shoulder" in the same region as transcobalamin II, though the bulk of radioactivity remained at the origin.

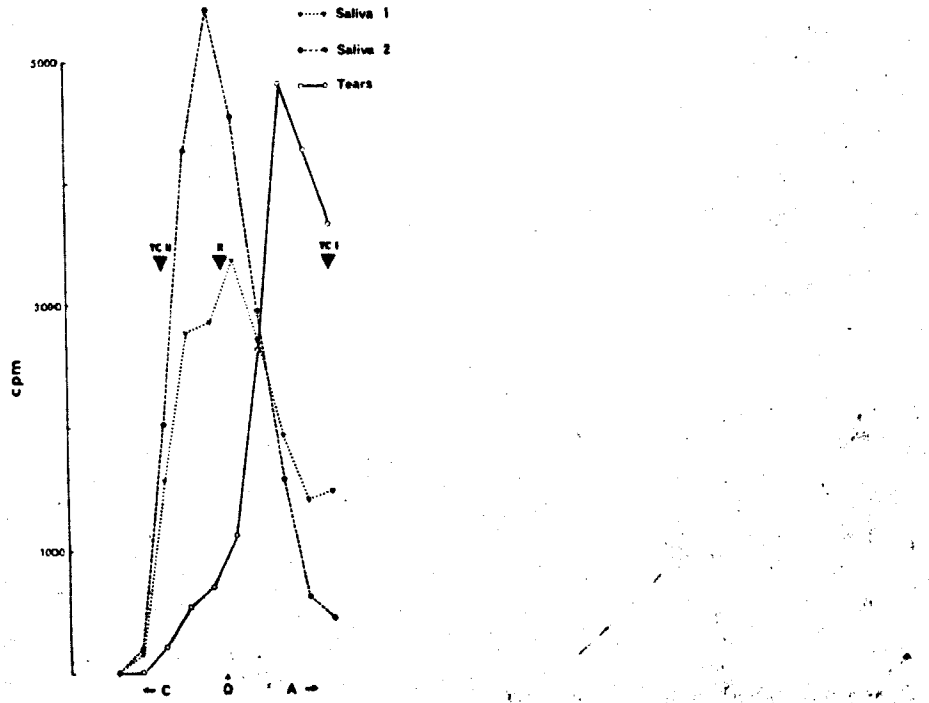

Fig. 2. Pevikon block electrophoresis at pH 4.5. Each point represents a 1-cm section. C = cathode; O = origin; A = anode. Migration of each of the three serum binders is indicated (transcobalamin II, R and transcobalamin I); leucocyte binder peak coincided with that of saliva 1.

Tear binder was identical to saliva binder on gel filtration. However, electrophoresis at pH 8.6 showed diffuse α_1–α_2 globulin mobility, and at pH 4.5 showed anodal migration intermediate between that of third binder (and leucocyte and saliva binders) and that of transcobalamin I (Fig. 2).

DISCUSSION

Filtration characteristics of proteins on gel reflect molecular radius rather than molecular weight, and estimates of the latter from gel filtration may be incorrect[17]. Overestimation may occur particularly with the less globular glycoproteins[18], such as vitamin B_{12} binders. Setting this important consideration aside, gel filtration results correspond to an estimated weight of 120 000 for all R binders and transcobalamin I (refs 2, 8, 10, 13 and 19).

Using a diffusion cell technique, Gräsbeck[4] reported such a figure for saliva binder. However, he subsequently calculated from gel filtration and ultracentrifugation data a molecular weight of 60 000 for the protein[5], making it unique among all R

Sephadex G-200 gel filtration, whether excess or subsaturating amounts of [^{57}Co]-vitamin B_{12} were used. The binder peak coincided with serum third binder and transcobalamin I peaks and with leucocyte binder peak, and eluted before serum albumin (Fig. 1). Saliva filtered without added [^{57}Co]vitamin B_{12} behaved identically, though about half the vitamin B_{12}-binding protein was lost or destroyed during filtration (Fig. 1). Filtration through Sephadex G-75 resulted in identical elution relationships, but the R binder and transcobalamin I peak always eluted two tubes after the void fraction.

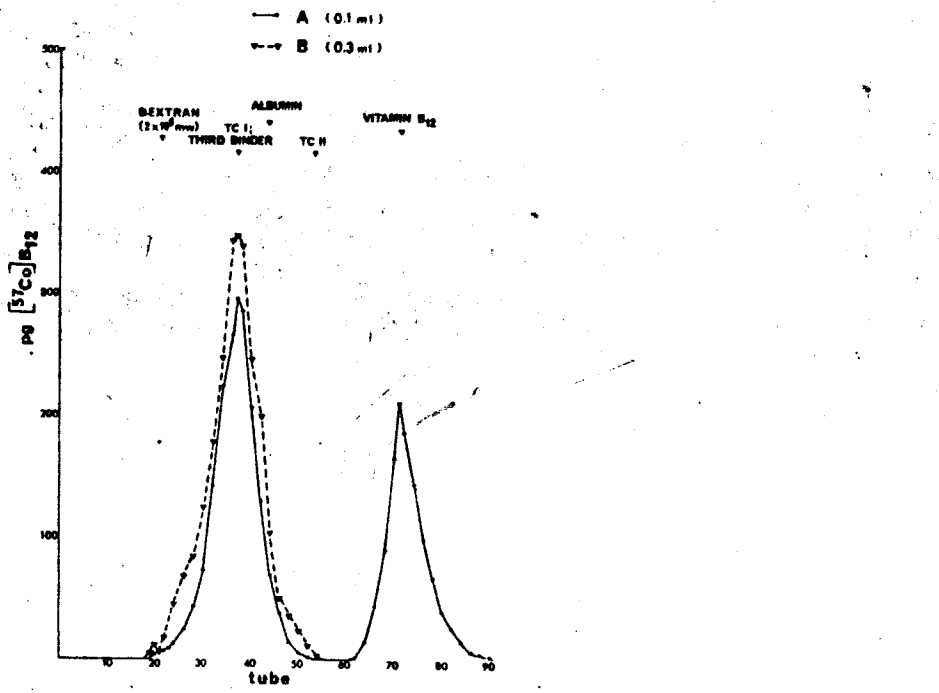

Fig. 1. Sephadex G-200 gel filtration of saliva. (A) Saliva filtered after saturation with [^{57}Co]-vitamin B_{12}; (B) saliva filtered before adding [^{57}Co]vitamin B_{12}. Tear and leucocyte binder peaks coincided with the transcobalamin I-third binder peak. The peak at Tube 71 is unbound [^{57}Co]vitamin B_{12}. TC = transcobalamin; mw = molecular weight.

Assuming a partial specific volume ($\bar{v}$) of 0.725 cm$^3 \cdot$g^{-1} (ref. 16), saliva binder, serum third binder and transcobalamin I had a mean sedimentation coefficient of 4.6 ± 0.3 (S.D.) when compared with the standards. Using these figures, calculations as outlined by Siegel and Monty[17] give an estimated molecular weight of 96 000 for the three binders. However, assumed $\bar{v}$ may be substantially incorrect.

Electrophoresis at pH 8.6 showed varying patterns. Specimens from all subjects had β-globulin mobility or just a bit faster. However, a second sample from one of the subjects showed a wide band of radioactivity encompassing α_2- and β-globulin regions, whereas a second from another subject had α_2-globulin mobility. In neither case was there significant quantitative change in vitamin B_{12} binding.

At pH 4.5, binder migration of two saliva specimens was minimal, resembling

binders tested in his laboratory[2]. Hurlimann and Zuber[6], using gel filtration alone, reported a weight of 60 000 also. However, they also found leucocytic, gastric and serum vitamin B_{12} binders to behave thus (and found only one serum binder molecular size), all eluting with or after albumin, which is at variance with all other reports[8,10–13,19–21].

The present data show that saliva binder is identical in molecular size and sedimentation characteristics to other R binders and transcobalamin I. Sucrose density gradient ultracentrifugation of saliva binder and transcobalamin I here gave a mean sedimentation coefficient of 4.6, compared with a value of 3.5 given the former by Gräsbeck and Visuri[5]. These values, however, and calculation of molecular weight from them, assume a $\bar{v}$ which may be incorrect. Therefore, the exact molecular weight of all vitamin B_{12} binders still awaits definition, but appears to be smaller than current estimates, a fact also suggested here and previously[20] by their slight retardation on Sephadex G-75 gel.

The reason for the discrepancy with the previous studies of saliva binder size by gel filtration is unclear. The protein may exist as a dimer, as may indeed other R binders. However, saliva binder elution was identical with and without [^{57}Co]vitamin B_{12}, indicating that this binding process, unlike vitamin B_{12} binding by intrinsic factor[3], does not itself cause dimerization. Alternatively, the extensive preparatory manipulation and chromatographic purification in the previous studies[5,6] may have altered the protein in size (as noted with transcobalamin II by Puutula and Gräsbeck[21]) or in shape. Vitamin B_{12} binders are susceptible to alteration[8], perhaps more so when not protected by being bound to the vitamin[22,23] or when exposed to very dilute solutions[8], both of which occurred in one of the studies[6]. Therefore, manipulation of samples was kept to a minimum in the present study. In several instances, testing was done directly within minutes of collection.

While saliva binder behaves as a single protein in molecular size, binding curve[24], and immunodiffusion studies[25], some heterogeneity obviously exists[1,5,6]. Charge variation may explain why the bulk of saliva binder, unlike gastric R binder, was eluted with the α-globulin fraction on rapid DEAE-cellulose chromatography[22]. Particularly noteworthy were the different electrophoretic patterns on different days in the same subject here, suggesting that the differences are due to acquired change, rather than to different genetic forms. Heterogeneity is not confined to saliva binder, and is demonstrable in leucocyte binder[9] and tear binder. The latter had been found to have predominantly α_2-globulin mobility[14] but here migrated in a broad band covering the α_1–α_2 globulin area. Its lower isoelectric point was further indicated by electrophoresis at pH 4.5 (Fig. 2).

It thus appears that R binders from different sources share all known characteristics save minor variations in charge, which, as has been suggested[1,8] may result from different sialic acid content. Transcobalamin I may be a more marked example of this variation, which may even play a role in its carrying endogenous vitamin B_{12}. However, the evidence indicates that serum third binder, not transcobalamin I, is the R binder in serum.

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VITAMIN B_{12} UPTAKE BY HUMAN SMALL BOWEL HOMOGENATE AND ITS ENHANCEMENT BY INTRINSIC FACTOR

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Mucosal homogenates of human ileum were demonstrated to have enhanced vitamin B_{12} uptake in the presence of normal human gastric juice or hog intrinsic factor concentrate. This process was demonstrated to require Ca^{++} or Mg^{++} and to be optimal at pH 6.6 and above. The enhancement was blocked by antibody to intrinsic factor. Pernicious anemia gastric juice and normal and pernicious anemia serum did not enhance B_{12} uptake. In fact, these substances diminished nonspecific uptake of the vitamin, presumably by binding it and rendering it unadsorbable. "Enhancement" by saliva appeared to be an artifact due to the presence of B_{12} binders precipitable by centrifugation. Human jejunal mucosa homogenate uptake was not enhanced by intrinsic factor, supporting the concept that the active process of vitamin B_{12} uptake is localized to the ileum. Nonspecific uptake of vitamin B_{12}, unrelated to intrinsic factor, appeared to be uniform in jejunal and ileal mucosa homogenates, and to be unaffected by *p*H or cations. Poor vitamin B_{12} uptake in some but not other ileal homogenates from patients with regional ileitis suggests that the ileal homogenate technique may be useful in evaluating in vivo function of intestinal receptors for the intrinsic factor-vitamin B_{12} complex. This awaits further studies, correlating this technique with in vivo absorption studies.

Vitamin B_{12} absorption takes place by two mechanisms.[1] The first is passive and nonspecific, taking place along the entire length of the intestine.[2] The second is the active mechanism which is the specific one mediated by intrinsic factor. The marked observable difference between these two mechanisms has formed the basis of in vitro techniques for study of

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the relation of intrinsic factor to vitamin B_{12} adsorption to the mucosal cell, the first step in the absorptive process. These techniques have been utilized on various animal liver[3-6] and intestinal[7-14] preparations. Studies with human liver have also been reported.[5, 15, 16]

The purposes of the present study are to establish with human intestinal homogenates various conditions required for vitamin B_{12} uptake, to help explain various clinical observations on the process, and to establish a basis for further study of the intestinal phase of the process by this technique.

Materials and Methods

Human ileum was obtained at surgery from patients undergoing resection for carcinoma of the ascending colon, ulcerative colitis, or regional enteritis. Specimens from patients with carcinoma of the colon, or, in a few instances, ulcerative colitis, were referred to as normal ileum. Jejunum was obtained from patients undergoing gastroenterostomy for ulcer disease. Immediately after resection, the intestinal segment, usually measuring 1 to 6 inches, was placed in cold (4 C) 0.85% NaCl (saline). The mucosal surface was washed with cold saline until the effluent was clear. Mucosal scrapings were obtained by exposing and scraping the mucosal surface, or by running a spatula along the serosal surface and, with firm pressure, expressing mucosal tissue as toothpaste is expressed from a tube. The scrapings thus obtained were placed in 50 ml of cold saline and homogenized in a Waring Blendor for 15 sec. The homogenate (0.5 to 5.0 g wet weight) was divided into aliquots and centrifuged for 10 min at 2000 rpm at 4 C. The pellets, washed twice with 5 ml of cold saline, were stored at −20 C until used. The ability of the homogenate to function in our assay system was maintained on such storage for as much as 1 year. Once thawed, specimens were used or discarded.

Human gastric juices were obtained from normal subjects, as well as from subjects with pernicious anemia or other conditions, and were depepsinized and neutralized.[13] Intrinsic factor content was assayed by the method of Gottlieb et al.[17] With the one exception noted below, only specimens containing intrinsic factor content of at least 80% of the total vitamin B_{12} binding capacity were used.

Saliva was obtained from various subjects by expectoration, occasionally with the aid of Parafilm chewing. Secretion of H substance was determined with *Ulex europaeus*.[18] Samples were stored at −20 C.

Antibody to human intrinsic factor was obtained from patients with pernicious anemia. Presence of the antibody was determined as reported elsewhere,[19] and the samples were stored at −20 C.

Hog intrinsic factor concentrate was purchased from the National Formulary of the American Pharmaceutical Association, Washington, D.C.

Co^{57}-labeled vitamin B_{12} ($Co^{57}B_{12}$) of specific activity 10 μc per μg was usually used; in some experiments $Co^{57}B_{12}$ of specific activity 5.9 μc per μg was used, as well as $Co^{60}B_{12}$ (kindly supplied by Dr. Elmer Alpert of Merck, Sharpe and Dohme Research Laboratories, West Point, Pa.) of specific activity 1.283 μc per μg. All were diluted with saline to a concentration of 10 ng per ml.

Rabbit anti-hog intrinsic factor concentrate antiserum was obtained from rabbits immunized with hog intrinsic factor concentrate as previously described.[19] The presence of antibody was assayed as with human serum.

For 30 min 0.2 to 0.5 ml of $Co^{57}B_{12}$ was pre-incubated at room temperature with 0.05 to 0.1 ml of gastric juice, made up to a total volume of 1.0 ml with Krebs Ringer-Tris (KRT) buffer, *p*H 7.4. Volumes used were such that the B_{12} binding capacity of the specimen equaled, or slightly exceeded, the amount of $Co^{57}B_{12}$ present. Controls with saline substituted for the gastric juice were used with each specimen assayed. On occasion, saliva or serum was used in place of the gastric juice. Gut pellets were thawed, weighed, suspended in cold KRT buffer, and homogenized using a motor-driven Teflon pestle with a fitted glass tube immersed in ice. The homogenate in buffer was constantly shaken to ensure uniformity of suspension, and equal 3-ml aliquots were added to each gastric juice-$Co^{57}B_{12}$ sample and to control samples. (Each tube in all of our experiments thus contained 25 to 100 mg of gut homogenate; best results were obtained when at least 60 mg was used. Samples of 15 mg proved too small to produce significant results.) As additional controls, gut homogenate was left out of the system in samples otherwise prepared identically to the test samples, and run concurrently in each assay. The samples were agitated at 90 cycles per min for 90 min at 37 C in a Dubnoff

Metabolic Shaker. Subsequently, 30-min agitation at room temperature was substituted when it was shown to produce even better results. The reaction was terminated by centrifugating at 2000 rpm for 10 min at 4 C. The precipitate was washed with 5 ml, and then with 3 ml, cold 0.85% NaCl-10, mM $CaCl_2$. Radioactivity of the pellet was determined in a well-type scintillation counter, for a minimum of 10,000 counts. The counts were converted to picograms of $Co^{57}B_{12}$. Each specimen was run in duplicates and differences between these were generally less than 10%. Ratios of $Co^{57}B_{12}$ uptake by the test specimens (S) over uptake by the saline control (C) were determined (S/C).

In experiments of cation dependence, $CaCl_2$ and $MgSO_4$ were omitted in preparing the KRT buffer, and saline, rather than saline-$CaCl_2$, washes were used. The effect of ethylenediaminetetraacetate (EDTA) was studied using 40 mg sodium versenate (Riker) per sample, diluting it 1:20 in KRT buffer from which $CaCl_2$ and $MgSO_4$ had been omitted, and adjusting the final *p*H of the specimen to 7.4 with 0.5 N NaOH. In studying *p*H dependence, the buffers used consisted of 9 parts Krebs Ringer solution[20] brought to appropriate *p*H with 1 part 0.05 M Tris-acid maleate-NaOH buffer.[21]

Results

Uptake by Normal Ileal Mucosa Homogenate

In the absence of intrinsic factor there was some vitamin B_{12} uptake by ileal homogenate. This represented nonspecific uptake and ranged from 10 to 20 pg, occasionally being as much as 35 pg, B_{12} per 100 mg homogenate. No more than 4 pg of $Co^{57}B_{12}$ was spun down when buffer alone instead of homogenate suspension was added. As demonstrated by the S/C ratios in figure 1, normal gastric juice markedly enhanced vitamin B_{12} uptake. The magnitude of the S/C ratio seemed to correlate primarily with the amount of gut homogenate used, the smaller ratios being seen with the smaller pellets. A given pellet of a single gut homogenate produced comparable results with different intrinsic factor-containing gastric juices. Significant enhancement was seen with gastric juices of normal persons and of patients with duodenal ulcer, tropical sprue, or iron deficiency. While these specimens all had high intrinsic factor content, even a specimen from a patient with tropical sprue where only 40% of the binding capacity was due to intrinsic factor markedly enhanced vitamin B_{12} uptake. Hog intrinsic factor concentrate had an identical effect, although perhaps of not so great a magnitude as the human gastric juices.

In marked contrast, pernicious anemia gastric juice not only did not increase uptake, but actually inhibited it. This was true with specimens from persons who secreted H substance as well as those who did not. Sera from normal and pernicious anemia subjects also resulted in reduced B_{12} uptake by the ileal homogenate (fig. 1).

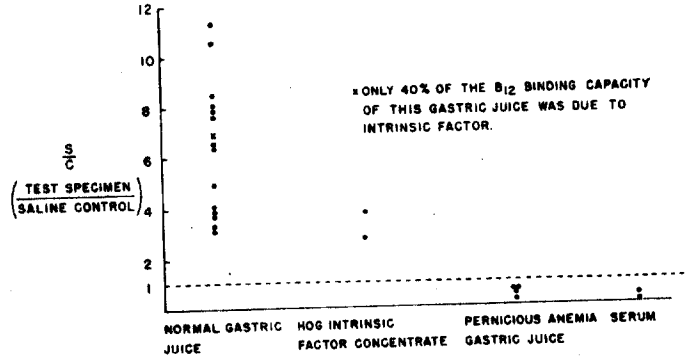

FIG. 1. Vitamin B_{12} uptake by normal ileal mucosa homogenate. (Points represent different mucosa specimens.)

Heating normal gastric juice at 100 C for 30 min (which destroys intrinsic factor activity) prior to testing it eliminated uptake enhancement, reducing an S/C ratio of 6.3 to 0.9.

Effect of Antibody to Intrinsic Factor

As shown in figure 2, intrinsic factor effect was eliminated when the normal gastric juice was incubated, prior to addition of $Co^{57}B_{12}$ or ileal homogenate, with antibody-containing serum from a patient with pernicious anemia, or if hog intrinsic factor concentrate was similarly preincubated with rabbit anti-hog intrinsic factor concentrate antiserum. A similar effect was noted when rabbit anti-hog intrinsic factor concentrate antiserum was used with human gastric juice. In contrast, sera without antibody had no effect.

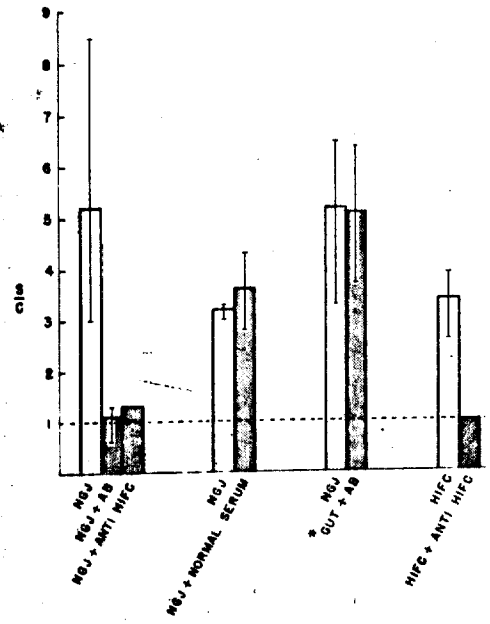

FIG. 2. Effect of antibody to intrinsic factor on vitamin B_{12} uptake by normal ileal mucosa homogenate. (Points represent different mucosa specimens.) Homogenate preincubated with human anti-intrinsic factor antiserum for 30 min at room temperature prior to incubation with normal human gastric juice and $Co^{57}B_{12}$. *S/C*, test specimen/saline control ratio; *NGJ*, normal human gastric juice; *AB*, human anti-intrinsic factor antiserum; *HIFC*, hog intrinsic factor concentrate; and *anti-HIFC*, rabbit anti-hog intrinsic factor concentrate antiserum.

That the antibody acts on the gastric juice binding of B_{12}, rather than on the gut itself, was shown by lack of effect of antibody incubated for 30 min at room temperature with the ileal homogenate (which was then washed with KRT) prior to addition of gastric juice and $Co^{57}B_{12}$.

Uptake by Jejunal Mucosa Homogenate

In contrast to ileal uptake, jejunal uptake of vitamin B_{12} was not enhanced by normal gastric juice (fig. 3). Similarly, pernicious anemia gastric juice had no effect.

Nonspecific uptake, as reflected by vitamin B_{12} uptake in the saline controls, did not differ significantly from that seen with ileal homogenates.

Uptake by Abnormal Ileal Mucosa Homogenates

With ileum from various patients with regional ileitis, intrinsic factor enhancement of vitamin B_{12} uptake was demonstrated in some cases (fig. 3). No enhancement was seen generally with those ileal specimens which had appeared grossly abnormal. In one case, however, two specimens from the same patient, one appearing normal to the naked eye and the other abnormal, were tested and both showed no intrinsic factor enhancement.

Effect of pH

The *p*H of our experimental system was 7.2. To determine *p*H effect on uptake by normal ileal homogenate, a *p*H range of 4.8 to 9.0 was studied. As shown in table 1, intrinsic factor effect was minimal or absent at *p*H 5.5 and absent below that level. Maximal effect was noted at *p*H 6.6 and above. Nonspecific uptake was not affected by *p*H change.

Effect of Ca^{++} and Mg^{++}

The intrinsic factor effect of gastric juice was shown to require divalent cation. Omission of both Ca^{++} and Mg^{++} abolished intrinsic factor enhancement (table

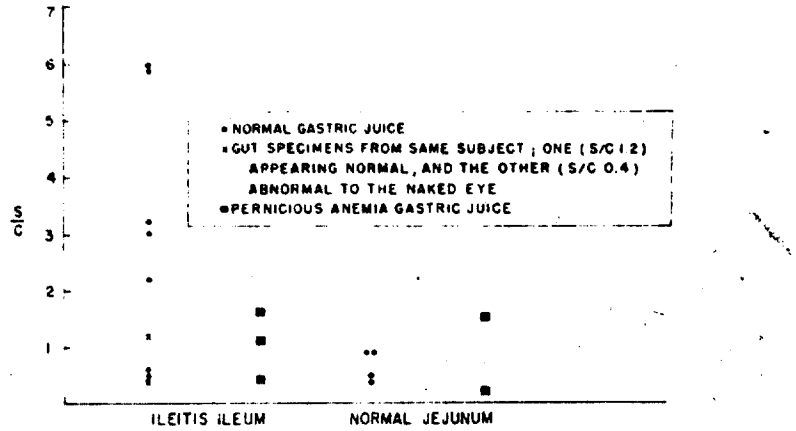

FIG. 3. Vitamin B_{12} uptake by ileal mucosa homogenate from patients with regional ileitis, and by normal jejunal mucosa homogenate. *S/C*, test specimen/saline control ratio.

2). This was not seen when Mg^{++} alone was omitted from the system. When Ca^{++} alone was left out, intrinsic factor effect was abolished with three of four normal gastric juices tested. The one exception demonstrated reduced, albeit still significant, intrinsic factor effect. This gastric juice was then further tested with sodium versenate (EDTA), which abolished its effect entirely (table 3). Addition of equimolar amounts of either $CaCl_2$ or $MgSO_4$ to the EDTA re-established intrinsic factor effect. In no instances was there artifactual precipitation of $Co^{57}B_{12}$. In previous experiments, EDTA was shown not to interfere with binding of $Co^{57}B_{12}$ by gastric juice. Nonspecific uptake of B_{12} was not affected by either omission of cations or by addition of EDTA.

Effect of Saliva

At *p*H 7.2, only three of eight saliva specimens increased B_{12} "uptake" by ileum homogenate, with an average S/C of 5.2. Two of these were from normal blood group A secretors of H substance, and one was from a blood group O nonsecretor with pernicious anemia. The 5 other subjects studied, regardless of secretor status or presence of pernicious anemia, gave negative results (average S/C of 1.4).

The three "positive" saliva specimens, however, produced identically large B_{12} precipitates when tested with jejunal and colonic mucosa, and, indeed, when tested with buffer alone without homogenate. Antibody to intrinsic factor had no effect

TABLE 1. *Effect of pH on intrinsic factor-mediated vitamin B_{12} uptake by normal ileal mucosa homogenate*

Experiment 1		Experiment 2	
*p*H	S/C[a]	*p*H	S/C[a]
4.9	0.1	4.8	0.5
5.5	0.1	5.5	2.1
7.0	3.5	6.6	6.3
7.8	4.7	7.5	3.7
8.5	4.3	9.0	5.2

[a] Expressed as test specimen to saline control ratio.

TABLE 2. *Effect of Ca^{++} and Mg^{++} lack on intrinsic factor-mediated vitamin B_{12} uptake by normal ileal mucosa homogenate*[a]

Base line	Absence of Ca^{++} and Mg^{++}	Absence of Ca^{++}	Absence of Mg^{++}
3.8	1.3		
4.9	1.4		
6.0		4.0	6.4
9.6		1.5	10.2
8.3		1.3	
8.1		1.8	

[a] Expressed as test specimen to saline control ratio.

TABLE 3. *Effect of sodium versenate (EDTA) on intrinsic factor-mediated vitamin B_{12} uptake by normal ileal mucosa homogenate*

	S/C[a]
Gastric juice	2.3
Gastric juice + EDTA	0.3
Gastric juice + EDTA + Ca^{++}	6.6
Gastric juice + EDTA + Mg^{++}	5.6
Gastric juice + EDTA + Ca^{++} + Mg^{++}	4.3

[a] Expressed as test specimen to saline control ratio.

on results with ileal or jejunal homogenate.

When the saliva was centrifuged at 4000 rpm for 15 min and only the supernate used, the positive effect with ileal mucosa or with buffer alone disappeared (S/C of 0.3). When the *p*H of the system was changed to 9.0, it was found that even salivas negative at *p*H 7.2 produced large precipitates of vitamin B_{12}.

Discussion

In vivo studies in man and both in vitro and in vivo animal studies suggest that vitamin B_{12} absorption occurs by two mechanisms,[1] passive transport and an active process which is dependent on intrinsic factor[1, 22, 23] and is limited to the ileum.[23–30] The results of the present in vitro study demonstrate the role of intrinsic factor in the uptake of vitamin B_{12} by human ileal mucosa.

Neither serum, saliva, nor pernicious anemia gastric juice or heated normal gastric juice increase vitamin B_{12} uptake. In fact, these substances even interfere with passive uptake, probably because they bind the vitamin, rendering it unavailable to the gut mucosa. Hog intrinsic factor concentrate resembles normal human gastric juice in this system.

Human auto-antibody to intrinsic factor, obtained from patients with pernicious anemia, and anti-hog intrinsic factor concentrate antiserum, prepared in rabbits, both block intrinsic factor effect on the gut homogenate.

The intrinsic factor effect was demonstrable on ileal but not jejunal mucosa homogenates. A previous study[31] of vitamin B_{12} uptake along the entire length of small intestine, obtained within 2 hr after death from a patient who died of acute leukemia, showed consistent enhancement by normal gastric juice of vitamin B_{12} uptake by mucosal homogenates of all segments from the distal half. Segments from the proximal half of the small intestine did not demonstrate this effect. We have not been able to repeat this study successfully, probably due to the rapid postmortem autolysis of intestinal mucosa which makes it mandatory that the specimen be obtained within 2 hr of death. Similar autolysis probably explains the better results obtained in the present study when incubation was carried out for 30 min at room temperature instead of 90 min at 37 C.

Intrinsic factor action was shown to be dependent on divalent cations, as had been demonstrated in vivo in man,[5, 32, 33] and in vitro in animals.[7, 8, 14, 34] Omitting both Ca^{++} and Mg^{++} from our system abolished intrinsic factor effect of all gastric juice. Omission alone produced no change, Ca^{++} omission did. EDTA completely abolished the intrinsic factor effect of all gastric juice. Adding either Ca^{++} or Mg^{++} alone to the EDTA re-established activity, suggesting that either cation was effective. It is probable that endogenous Ca^{++} and Mg^{++} in the gastric juice and gut homogenate accounts for the discrepancies in some cation omission experiments.

Intrinsic factor was maximally effective in the range of *p*H 7, minimally effective at *p*H 5.5, and ineffective below that level, similar to guinea pig ileal homogenates.[13]

In contrast to the intrinsic factor-mediated process, the nonspecific uptake of vitamin B_{12} does not seem to be affected by *p*H or divalent cations and to occur in jejunal as well as ileal mucosa.

Enhancement by some salivas containing H substance of vitamin B_{12} uptake by guinea pig ileal homogenate, which had been reported to occur independently of intrinsic factor,[2] was not demonstrated with human ileal homogenate. Three sa-

liva specimens of the eight tested seemingly enhanced vitamin B_{12} uptake. However, this effect was artifact, as demonstrated by the equally large vitamin B_{12} precipitates with jejunal and colonic as compared with ileal mucosa homogenates, and even on incubation without gut homogenate. Antibody to intrinsic factor had no effect on saliva. In addition, a nonsecreting patient with pernicious anemia was fed $Co^{57}B_{12}$ with 250 ml of saliva from two blood group O secretors of H substance, in a Schilling test to determine whether the saliva enhanced B_{12} absorption. Urinary excretion in a 24-hr period was negligible (1.8%). The precipitates with saliva are probably a manifestation of a precipitable component, possibly mucus.[34] This serves to emphasize the need for a control consisting of the test samples without gut homogenate, or with added EDTA, which would eliminate effect of intrinsic factor, but not effect of artifact.

It has been postulated that receptor sites exist on the ileal mucosal cell surface for the intrinsic factor-B_{12} complex.[2, 35, 36] Depressed vitamin B_{12} absorption has been reported in patients with regional ileitis.[36] Our data on ileal homogenates from patients with this disease show that vitamin B_{12} uptake is subnormal in some cases, but not in others, and that this need not be related to the gross appearance of the mucosa to the naked eye. This suggests that this technique may be useful in examining the functional status of the receptors on the mucosal cell for the intrinsic factor-B_{12} complex. However, further studies, correlating this technique with in vivo absorption studies, are required.

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[202] Spectrophotometric Determination of CoB_{12} in the Presence of B_{12} Compounds

By DORIANO CAVALLINI and ROBERTO SCANDURRA

Principle

Cyanocobalamin and hydroxocobalamin (both indicated as B_{12}) in the presence of alkaline cyanide are converted into dicyanocobalamin at a fast rate. Under the same conditions deoxyadenosylcobalamin (CoB_{12}) is converted into dicyanocobalamin at a much slower rate. The difference of extinction at a suitable wavelength before and after treatment with alkaline cyanide gives the total content of CoB_{12} and B_{12}, while the kinetics of the conversion reaction allows one to establish the ratio CoB_{12}:B_{12}.

Assay Method

The following method comes from the work of Cavallini and Scandurra[1] and Scandurra, Marcucci, and Ferretti.[2]

Reagents

Glycine–NaOH buffer, 5×10^{-2} *M*, pH 10

Glycine–NaOH–KCN buffer. To the glycine–NaOH buffer 5×10^{-2} *M*, pH 10, solid KCN is added to the final concentration of 0.2 *M*. The solution is then adjusted to pH 10 with few drops of 2 *N* HCl.

Procedure. Spectrophotometric cuvettes with a light path of 1 cm and a content of 1 ml of solution are used. The determination is carried out in a room illuminated with a faint red light. Into one cuvette, 0.5 ml of the test solution containing 20–50 μg of CoB_{12} and 0.5 ml of glycine–NaOH buffer are pipetted. The mouth of the cuvette is covered with parafilm, and the contents are mixed by 2–3 inversions. The extinction at 580 nm against water gives the value E_0. Into another cuvette, 0.5 ml of the same test solution and 0.5 ml of glycine–NaOH–KCN buffer are pipetted. As soon as the alkaline solution is added, a chronometer is started and the contents of the cuvette are mixed as above. Readings against water at 580 nm are taken every 2 minutes for the first 20 minutes. This gives the values E_t at different times. The cuvette is then left in the light and readings are

[1] D. Cavallini and R. Scandurra, *Acta Vitaminol. Enzymol.* **21**, 5 (1967).
[2] R. Scandurra, M. Marcucci, and M. G. Ferretti, *Acta Vitaminol. Enzymol.* **21**, 165 (1967).

taken again after 2 hours and 2.5 hours from the start. In general, these two values are very similar and give the value E_∞.

Calculation

The value $E_\infty - E_0$ gives the total amount of coenzymatic and non-coenzymatic cobalamins present in the sample. The percentage of this value due to the presence of CoB_{12} is calculated by extrapolation to zero time the E_t values plotted in a semilogarithmic graph as reported in Fig. 1. The extinction due to CoB_{12} is obtained by:

$$\Delta E_{CoB_{12}} = (E_\infty - E_0) \times (C/100)$$

where C is the percentage obtained from the graph. The amount of CoB_{12} in the sample is calculated by:

$$\mu g\ CoB_{12} = \Delta E_{CoB_{12}} \times 196.3$$

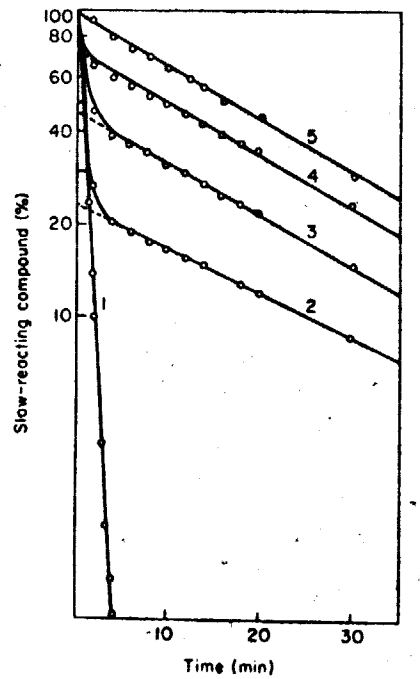

FIG. 1. Semilogarithmic plot of the values E_t after treatment of mixtures of deoxyadenosylcobalamin and cyanocobalamin with alkaline cyanide. Mixtures containing hydroxocobalamin instead of cyanocobalamin behave similarly. The percentage of CoB_{12} in the mixtures was as follows: curve 1, 0%; curve 2, 25%; curve 3, 50%; curve 4, 75%; curve 5, 100%.

where 196.3 represents the difference of extinction $E_\infty - E_0$ at 580 nm of pure CoB_{12} ($\Delta E_{1cm}^{10^{-5}M} = 8.05$).

Comment

The method has been checked with pure compounds and found satisfactory in the range 20–50 μg CoB_{12} in the cuvette. It has been used also with impure extracts provided that the accompanying impurities do not absorb at 580 nm and do not change extinction appreciably at this wavelength in the presence of glycine–NaOH–KCN buffer. Although other wavelengths could be used to follow the change into dicyanocobalamin, the 580 nm wavelength has been chosen to minimize disturbance by ultraviolet absorbing compounds. The enzymatic method[3] is much more sensitive than that described. However, the spectrophotometric method may represent a suitable alternative when the sensitivity of the enzymatic method is not necessary. It is very convenient, for instance, for checking the purity of crystalline CoB_{12}.

Acknowledgment

This work has been supported by a grant from the Consiglio Nazionale delle Ricerche.

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Ind. Jour. Med., Res., **45**, 3, July, 1957.

STUDIES IN EXPERIMENTAL ATHEROSCLEROSIS.

Part V.

THERAPEUTIC EFFECT OF ASCORBIC ACID AND VITAMIN B_{12} IN CHOLESTEROL ATHEROSCLEROSIS.

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INTRODUCTION.

VITAMINS, which are essential dietary factors, play a significant rôle in the intermediate metabolism of lipids but enough information is not available of their effect in atherosclerosis (McHenry and Cornett, 1944). Myasnikov (1950, 1954) reported that continued administration of ascorbic acid in cases of human atherosclerosis lowered cholesterolæmia. Ascorbic acid is also known to be responsible for the formation of ground substance of arterial intima (Willis and Fishman, 1955) and its deficiency produced typical atheromatous lesion in guinea-pigs, a species which is otherwise fairly resistant to experimental production of atheroma (Willis, 1953). The same authors (Willis and Fishman, *loc. cit.*) also found localized depletion of ascorbic acid in atheromatous arteries and reported that incorporation of radio-active acetate in tissue cholesterol is several times more rapid than normal in tissues depleted of ascorbic acid.

Vitamin B_{12} is another dietary factory which has a direct rôle in the metabolism of fat, especially in the synthesis of methyl groups leading to the formation of choline and methionine in the body (Smith, 1956). Again, deficiency of vitamin B_{12} causes inactivation of SH enzymes and results in the accumulation of fat in tissues and thus this vitamin can act as a lipotropic agent (Smith, *loc. cit.*). Its rôle in atherosclerosis has so far not been investigated.

In view of the above, it was felt that administration of ascorbic acid and vitamin B_{12}, alone and in combination, may prove helpful in the prevention of atheromatous lesions experimentally produced in rabbits. The effectivity of this therapeutic regimen has been assessed biochemically and histologically.

MATERIAL AND METHODS.

Male rabbits of the C.D.R.I. colony, twenty-four in number, average weight 1·27 kg., were devided into four groups of six each and fed 0·5 g. cholesterol (Merck) suspended in 5 c.c. olive oil daily in addition to stock diet. Group 1 was kept as control. Groups 2, 3 and 4 received daily intramuscular injections of Redoxon (Roché) 50 mg., Macrabin (Glaxo) 10 µg. and a combination of both, respectively. The animals were observed for a period of twelve weeks after which they were sacrificed.

Biochemical estimation of total serum cholesterol and fractions (Schoenheimer and Sperry, 1934) and serum lipid phosphorus (Youngburg and Youngburg, 1930) were performed. Naked eye examination of lungs, liver, kidneys and heart together with aorta, pulmonary artery and other vessels was made. The severity and extent of aortic atheroma was visually graded (Chakravarti *et al.*, 1956). Histochemical and histological examination of tissues was made according to the procedure followed by Chakravarti *et al.* (1955).

RESULTS.

Biochemical.

TABLE I.

Mean values of serum cholesterol in control and treated cholesterol-fed rabbits.

Group.	Total cholesterol, mg./100 c.c.	Cholesterol ester, mg./100 c.c.	Free cholesterol, mg./100 c.c.	Lipid phosphorus, mg./100 c.c.	F/C.	C/P.
I	412±23	300±21	112±11·5	14 ±5·5	0·27	29·4
II	356±40	358±24	98±30·5	17·5±4·5	0·27	20·0
III	366±58	200±35	166±31	16·8±3·5	0·45	21·7
IV	376±41	196±32	180±35	16·5±4	0·48	22·7

Key:—

$$F/C = \frac{\text{Free cholesterol.}}{\text{Total cholesterol.}} \qquad C/P. = \frac{\text{Total cholesterol.}}{\text{Lipid phosphorus.}}$$

Table I shows that the total serum cholesterol attains highest score in group I animals, while the values are, more or less, similar in the other three groups. Free cholesterol is highest in group IV and least in group II, while group I and III lie in between. The ratio between free cholesterol/total cholesterol, F/C, is maximum in group IV almost double those in groups I and II, while group III shows a near approach to group IV.

The value of serum lipid phosphorus is similar in all four groups and the ratio of total cholesterol/lipid phosphorus, C/P, is most marked in group I and shows a steady decline in the other groups.

Histopathological.

TABLE II.

Grading of the severity and extent of atheroma of aorta of cholesterol-fed untreated and treated rabbits.

Animal number.	Group 1:			Group 2:			Group 3:			Group 4:		
	Ascending and arch.	Thoracic descending.	Abdominal.	Ascending and arch.	Thoracic descending.	Abdominal.	Ascending and arch.	Thoracic descending.	Abdominal.	Ascending and arch.	Thoracic descending.	Abdominal.
1	...	...	...	2	1	0	2	0	0	...	...	...
2	2	0	0	3	2	0	0	0	0	2	2	0
3	3	2	1	2	1	0	2	0	0	3	1	0
4	3	2	1	...	...	...	3	1	0	4	1	0
5	3	2	1	4	0	0	3	2	0	1	0	0
6	2	0	0	...	...	...	3	0	0	1	0	0
Mean	2·6	1·2	0·6	2·75	1·0	0·0	2·2	0·5	0·0	2·2	0·8	0·0
Mean per cent lesion per aorta ...		36·6			31·25			22·5			25·0	

Naked eye appearance.—From Table II it will be seen that visual grading of aorta showed maximum lesion in group 1, while groups 2, 3 and 4 registered a fair degree of fall. The heart usually showed atheromatous thickening of aortic and pulmonary valves and partial occlusion of coronary ostia, while large plaques of atheroma occupied the ascending and arch of aorta and pulmonary artery in group 1. The extent of disease was less marked in the other groups. But lesions in lungs, liver and kidneys were similar in all four groups.

Microscopic appearance.—Microscopic examination showed marked atheromatous involvement of aorta, pulmonary artery, branches of coronary, bronchial and in a few the interlobular arteries of kidneys in group 1 animals, while heart, lungs, liver and kidneys showed generalized fatty infiltration. The extent and severity of lesions was less marked in the other groups, especially so in group 4 in which minimal atheroma was noted in the coronary arteries.

DISCUSSION.

The present experiment shows that both ascorbic acid and vitamin B_{12} tend to lower the level of serum cholesterol in cholesterol-fed rabbits and vitamin B_{12} brings about a marked increase of free cholesterol as compared to cholesterol ester and increases F/C ratio. In experimental atheroma, it is the cholesterol ester which readily gets precipitated from the blood as a deposit in the intima of vessels, and, in this investigation, vitamin B_{12} appears to exert a protective measure by lowering the ester content of blood. On the contrary, ascorbic acid appears to have no effect on the F/C ratio and it has failed to produce similar protective effect

on the extent of atherosclerosis. Again, ascorbic acid and vitamin B_{12} appear to have much less effect on lipid phosphorus content of blood, thereby indicating that they do not exert their ameliorative effect on atheroma through an alteration of C/P ratio.

Flexner *et al.* (1941) reported that administration of ascorbic acid did not have any effect either on the serum cholesterol or on the extent of atheroma produced by cholesterol feeding in rabbits but Myasnikov (*loc. cit.*) obsevred that this vitamin brought down the blood cholesterol level appreciably and produced an inhibitory effect on experimental cholesterol atherosclerosis in rabbits, while Willis (*loc. cit.*) also noted similar effect in guinea-pigs. Our observations tend to support the findings of Myasnikov (*loc. cit.*) and Willis (*loc. cit.*). It is just possible that higher doses of this vitamin might have yielded better results.

The mechanism of action of vitamin B_{12} in cholesterol atheroma is not known. It may, however, exert its ameliorative effect by formation of lipotropic factors, such as choline and methionine in the body or it may have a specific action on cholesterol metabolism. It, however, opens a field for further investigations which may reveal a link between deficiency of this vitamin and the incidence of atherosclerosis.

SUMMARY.

1. Experimental atherosclerosis was produced in rabbits by feeding cholesterol in olive oil for a period of twelve weeks. During this period ascorbic acid and vitamin B_{12} injections, alone and in combination, were also given to different, groups of animals.

2. Vitamin B_{12} alone and in combination with ascorbic acid, produce greater degree of inhibition of atheroma than ascorbic acid alone.

3. Vitamin B_{12} markedly increased free cholesterol and $\frac{\text{Free cholesterol}}{\text{Total cholesterol}}$ ratio in serum.

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Brit. Med. J. 2(5811): 441-442

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RELIMINARY COMMUNICATIONS

ole of Third Serum Vitamin B_{12} inding Protein in Vitamin B_{12} ransport

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itish Medical Journal, 1972, 2, 441–442

ummary

third vitamin B_{12} binding protein present in normal rum has been shown to participate in transport of abelled vitamin B_{12} absorbed from the gut. All three itamin B_{12} binding proteins in serum were labelled at e same time after oral administration of vitamin B_{12}, mplying that "free" vitamin B_{12} reached the portal lood from the gut mucosa.

ntroduction

Two proteins in serum, transcobalamins I and II (T.C. I and II), are implicated in the transport of vitamin B_{12}. In the fasting state T.C. I carries almost all of the vitamin B_{12} present in plasma. Vitamin B_{12} entering the plasma is largely thought to be carried by the second binder, T.C. II. Hall and Finkler (1965) reported that after oral vitamin B_{12} T.C. II was the first vitamin B_{12} binding protein to be labelled. The T.C. II and vitamin B_{12} complex is rapidly cleared from plasma in a manner similar to haptoglobin (Hall and Finkler, 1965), and in conjunction with the short half-life of the T.C. II and vitamin B_{12} complex it has been suggested that T.C. II is concerned primarily with transport of vitamin B_{12} from gut to tissues. There is a lag of several hours before vitamin B_{12} in the gut appears in blood, and a possible explanation is that the delay is due to the synthesis of carrier protein (?T.C. II) in the intestinal cells.

Under certain conditions a third protein in plasma showing different chromatographic behaviour from either T.C. I or T.C. II is demonstrable. Hom (1967) and Cooper (1969) suggested that such a third vitamin B_{12} binding protein was a polymer of T.C. II. The monomer has a M.W. of about 35,000 and the polymer a M.W. above 120,000. Lawrence (1969) also identified three vitamin B_{12} binders, T.C. II being separable into low and high M.W. components. Gizis, Dietrich, Ohoi, and Meyer (1970) eluted three vitamin B_{12} binding fractions from serum on DEAE cellulose. The third binder linked to labelled vitamin B_{12} was injected intravenously and was cleared from plasma at a somewhat slower rate than T.C. II. A third vitamin B_{12} binder was described by Hall and Finkler (1967) in a patient with polycythaemia and a similar binder was also described by Carmel (1972) and Bloomfield and Scott (1972). It remains uncertain whether the third binder is a polymer of T.C. II or whether it is an antigenically distinct protein.

This study aimed to determine the physiological role of the third vitamin B_{12} binding protein, particularly in relation to the intestinal absorption of vitamin B_{12}, and to see whether this would shed any light on its nature.

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Materials and Method

^{57}Co-labelled vitamin B_{12} (20 μCi and 0·3 μg) was given by mouth to two volunteers over the age of 60. The procedure was explained and informed consent to the procedures given. Twenty-five ml of blood was obtained at the start of the procedure and at various times thereafter up to 48 hours. Plasma radioactivity was measured in all samples. Each plasma sample was concentrated twofold with an Amicon Diaflo ultrafilter before column chromatography.

T.C. I on the one hand and T.C. II and binder III on the other were separated on DEAE cellulose and the fractions containing T.C. II and binder III then placed on Sephadex G-200 (Bloomfield and Scott, 1972) to separate these two binders. Between 84% and 100% of the material put on the column was eluted from DEAE cellulose and between 56% and 96% from Sephadex G-200. (Figs. 1 and 2).

Results

No radioactivity was detected in plasma during the first two hours but was present at three hours. The distribution of the orally-administered dose among T.C. I, T.C. II, and binder III at this time is shown in the Table. The absorbed dose of vitamin B_{12} was attached to all three vitamin B_{12} binding proteins.

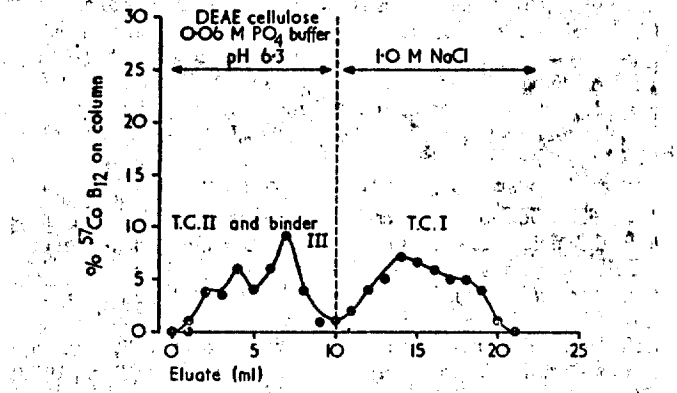

FIG. 1—Separation of vitamin B_{12} binding proteins on DEAE cellulose. T.C. II and a third binder were eluted with 0·06M phosphate at pH 6·3 followed by elution of T.C. I with 1·0M NaCl.

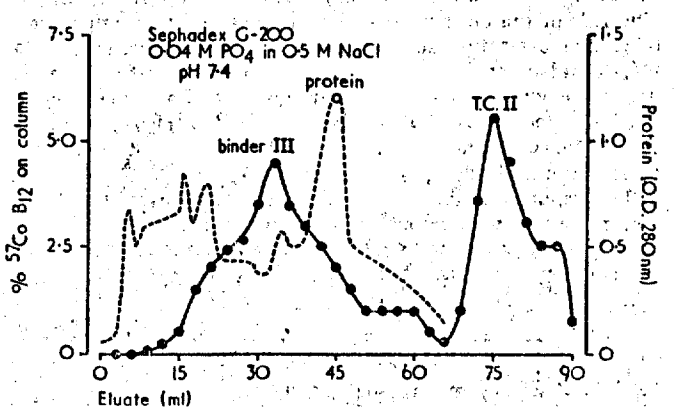

FIG. 2—Further chromatography of fractions containing T.C. II and Binder III on Sephadex G-200. Elution is with 0·04M phosphate in 0·5M NaCl at pH 7·4.

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Thereafter the amount attached to these fractions increased and was still present at 48 hours (see Table). Essentially similar results were obtained in both patients.

Relative Distribution of Vitamin B_{12} Absorbed from the Gut among Serum Vitamin B_{12} Binding Proteins

Time after Oral Dose of ^{57}Co Vitamin B_{12} (Hours)	Percentage of Oral Dose per Litre of Plasma			
	Whole Plasma	Transcobalamin		Binder III
		I	II	
1	0	0	0	0
2	0	0	0	0
3	0·44	0·23	0·13	0·13
4	1·80	0·78	0·64	0·17
5	3·26	1·32	0·68	0·28
6	3·25	1·58	0·73	0·15
24	2·58	0·85	0·21	0·18

Discussion

All three vitamin B_{12} binding serum proteins took up vitamin B_{12} absorbed from the gut and all three appeared to take it up at the same time. Thus this study failed to confirm the claim that T.C. II was the first binder to take up vitamin B_{12} absorbed from the gut. This finding makes it unlikely that the delay in transport of vitamin B_{12} from gut to blood is due to synthesis of a carrier protein and more likely that in the intestinal absorption of vitamin B_{12} the free vitamin passes to the blood. The absorbed vitamin B_{12} links equally to all the carrier proteins available and the total uptake by these proteins is related to their unsaturated vitamin B_{12} binding capacity at the time.

The third vitamin B_{12} binder participates in vitamin B_{12} transport under physiological conditions. Its plasma clearance when bound to vitamin B_{12} is not very dissimilar to T.C. II (Gizis *et al.*, 1970) and it remains to be shown that it is not a polymer of T.C. II.

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Further Studies on Vitamin B_{12} Deprivation and Carbohydrate Metabolism

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Vitamin B_{12} deprivation creates an elevated DPN/DPNH ratio in rat liver which can be correlated to the magnitude of B_{12} depletion. The slow response of the changed DPN/DPNH ratio to vitamin B_{12} repletion in deficiency indicates that vitamin B_{12} influences coenzyme I secondarily. The elevated DPN/DPNH ratio may be partly attributed to the decrease of activity in the reduced GSH due to B_{12} deficiency.

In B_{12}-deficient rats, an accumulation of blood pyruvic and lactic acids is observed in postabsorptive state when the body is at rest.

INTRODUCTION

In the vitamin B_{12}-deficient animals decreased GSH[2] activity in erythrocytes (1, 2) and elevated CoA levels in liver (3, 4) have been observed. These coenzymes are involved in carbohydrate metabolism, and the reported effects of the state of vitamin B_{12} nutrition on them prompted us to study further the possible role of the vitamin in other phases of carbohydrate metabolism. These studies deal with the effect of B_{12} deficiency on the hepatic DPN and DPNH, as well as on blood pyruvic and or lactic acid metabolism.

EXPERIMENTAL PROCEDURE

Animals and Diets

Rats of the McCollum strain were used. Vitamin B_{12} deficiency was induced by feeding the pregnant rats a BSD (5). From each litter, two or three weanling rats were kept on the vitamin B_{12} deficient BSD, while their litter mates were given the same diet supplemented with 100 μg. vitamin B_{12}/kg. feed. The latter group served as control. The composition of the BSD is: 70% commercial defatted soybean meal, 26% sucrose, 4% salts IV (6), and vitamin supplements (7).

Methods of Determination

(*a*) DPN and DPNH in liver were determined spectrophotometrically using alcohol dehydrogenase (8, 9). (*b*) The content of B_{12} in liver was estimated by a microbiologic assay using *Lactobacillus leichmannii* A.T.C.C. 1797 (10) as the test organism. (*c*) Blood levels of pyruvic and lactic acids in animals at least 10 weeks old were determined by the methods of Friedemann and Haugen (11) and Barker and Summerson (12), respectively. To minimize the effect of differences in food intake prior to the determination on blood pyruvic and lactic acid contents, the rats were fasted 16–18 hr. and then anesthetized with 2% sodium pentothal intraperitoneally. The chest cavity was opened and blood was drawn from the exposed heart within 2–3 min. after the injection and heparinized. The animals were kept as quiet as possible during the experiment to minimize undue alteration in blood lactic and pyruvic acid levels on account of activity or excitement.

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[2] Abbreviations used: GSH, reduced glutathione; CoA, coenzyme A; DPN, diphosphopyridine nucleotide; DPNH, reduced diphosphopyridine nucleotide; BSD, basal soybean diet; CoASH, reduced coenzyme.

RESULTS

Vitamin B_{12} Deficiency and DPN

Table I summarizes the data on liver DPN, DPNH, and B_{12} contents in the deficient and control animals. In seven pairs of female litter mates, the DPN concentration of liver was significantly increased and DPNH was greatly decreased in the deficient rats. The total DPN was only slightly and insignificantly increased. The ratio of DPN to DPNH was doubled as a result of deprivation, while the hepatic reserve of B_{12} dropped to less than 20% of the controls. Studies with a small number of male animals yielded similar results.

In order to rule out the possibility that an increased DPN/DPNH ratio may be associated with decreased feed intake and retarded rate of growth in B_{12} deficiency, pair-feeding experiments were conducted. The animals employed were litter mates. Our results (Table II) indicated that though the body weights of two groups of the animals were essentially equal, the ratio of DPN/DPNH of the B_{12}-treated animals was lower than that of the B_{12}-deficient animals.

Repletion with B_{12} and Supplementation of GSH in B_{12} Deficiency

The response of liver DPN, DPNH, and B_{12} levels of B_{12}-deficient rats to B_{12} treatment was investigated. Repletion was achieved by injecting the deficient animals daily with 3 μg. B_{12} intraperitoneally, and at the same time their diet was supplemented with 100 μg. B_{12}/kg. The rats were sacrificed at intervals of 5–10 days after the start of this regime, and the liver DPN, DPNH, and B_{12} contents were determined.

Since B_{12} deficiency results in a decrease of GSH in the erythrocytes (1, 2), it is of interest to study the correlation of GSH and DPN metabolism by determining whether the elevated ratio of DPN/DPNH of those deficient rats could be reduced by GSH administration. Therefore, in some of the repletion experiments, GSH was injected daily by the intraperitoneal route at 25 mg. dose per rat, together with vitamin B_{12} for the entire repletion period of 10 days. Another group of deficient rats received GSH only so as to evaluate the effect of GSH on DPN/DPNH ratio in B_{12} deficiency.

The results (Table III) indicate that

TABLE I

DPN, DPNH and Vitamin B_{12} Contents in Rat Liver

Number in parentheses denotes number of rats used.
Number in brackets denotes range.

Diet	Sex	Age	B.W.	DPN	DPNH	DPN + DPNH	$\frac{DPN}{DPNH}$	B_{12}
		weeks	*g.*		*μg./g. liver*			*mμg./g. liver*
$-B_{12}$	F	8–9	99 ± 6(7)	512 ± 14	147 ± 8	659 ± 15	3.6 ± 0.2	23 ± 4
$+B_{12}$	F	8–9	142 ± 5(7)	383 ± 28	213 ± 15	596 ± 36	1.8 ± 0.1	129 ± 9
P			<0.01	<0.01	<0.01	>0.05	<0.01	<0.01
$-B_{12}$	M	7–10	123(4)	[450–738]	[114–239]	[633–852]	[2.4–6.5]	[17–33]
				564	184	748	3.4	22
$+B_{12}$	M	7–10	171(2)	[364–410]	[205–217]	[581–615]	[1.7–2.0]	[93–102]
				387	211	598	1.8	98

TABLE II

DPN, DPNH and B_{12} Contents in Pair-Fed Rat Livers

Number in parentheses denotes number of rats used.

Diet	Sex	Age	B.W.	DPN	DPNH	DPN + DPNH	$\frac{DPN}{DPNH}$	B_{12}
		weeks	*g.*		*μg./g. liver*			*mμg./g. liver*
$-B_{12}$	F	13	172(2)	546	208	754	2.8	30
$+B_{12}$	F	13	173(2)	506	411	917	1.2	147

TABLE III

EFFECTS OF REPLETION AND GSH SUPPLEMENTATION ON LIVER DPN/DPNH AND B_{12} CONTENTS

Animals No.	Animals Age	Treatment[1]	Repletion period	DPN/DPNH Range	DPN/DPNH Average	Liver B_{12}
	weeks					*mμg./g.*
7	8–9	B_{12} deficient	8–9 wks.	2.8–4.7	3.6	23
3	8–11	B_{12} repleted	5 days	2.0–3.8	3.0	42
3	8–11	B_{12} repleted	10 days	2.0–2.9	2.4	84
7	8–9	B_{12} treated or repleted	4–5 wks.	1.2–2.3	1.8	129
6	8–9	stock diet	8–9 wks.	1.0–2.4	1.7	102
3	8–11	B_{12} repleted + GSH	10 days	1.6–2.0	1.9	86
2	8–11	B_{12} deficient + GSH	8–11 wks. 5–10 days	2.2–2.7	2.5	27

after 5 days of repletion with B_{12}, liver DPN/DPNH ratio dropped slightly as B_{12} levels increased. On 10 days of repletion, the ratio decreased further with concomitant increase in liver B_{12}. When the treatment was continued for 4–5 weeks, the ratio of liver DPN and DPNH and the hepatic reserve of B_{12} were restored to those of our stock rats.

These findings indicate that liver DPN/DPNH ratio increases according to the degree of vitamin B_{12} depletion. The correlation of these two phenomena was obtained from the straight line calculated by the regression method and is statistically significant with $p < 0.01$.

Animals receiving the daily supplementation of GSH and B_{12} for a period of 10 days appeared to have a lower average liver DPN/DPNH ratio (2.4 vs. 1.9) (Table III) than that of animals repleted with B_{12} alone. The liver B_{12} levels of the two groups were about equal. The daily administration of GSH alone to B_{12}-deficient rats for a period of 5–10 days, brought about a decrease of the liver DPN/DPNH ratio of 2.5 from 3.6 for the untreated group. As may be expected, their hepatic reserve of B_{12} was low.

VITAMIN B_{12} DEFICIENCY AND PYRUVIC ACID–LACTIC ACID RELATIONSHIP

The blood pyruvic acid and lactic acid contents of vitamin B_{12}-deficient rats were determined. As a measure of vitamin B_{12} deficiency, the vitamin B_{12} levels in liver were also assayed. The data of a total of six experiments consisting of 15 animals are

TABLE IV

EFFECT OF B_{12} DEPRIVATION ON BLOOD PYRUVIC AND LACTIC ACIDS AND B_{12} CONTENTS IN RAT LIVER

Number in parentheses denotes number of rats used.

Number in brackets denotes actual number.

Expt. No.	Pyruvic acid −B_{12}	Pyruvic acid +B_{12}	Lactic acid −B_{12}	Lactic acid +B_{12}	B_{12} contents −B_{12}	B_{12} contents +B_{12}
	mg. %		*mg. %*		*mμg./g. liver*	
1	4.0(1)	3.2(2)	28	18 [20, 17]	50	200
2	3.7(1)	2.6(1)	24	7	40	158
3	3.7(2)	1.2(1)	25 [29, 21]	18	40	212
4	4.7(1)	2.8(1)	23	11	46	111
5	4.0(2)	3.5(1)	28 [25, 31]	31	18	110
6	4.4(1)	3.5(1)	65	16	12	161
Mean	4.1 ± 0.03	2.8 ± 0.11	32 ± 6.62	17 ± 3.7	31 ± 6.4	160 ± 17.2
p	<0.05		<0.05		<0.01	

TABLE V

EFFECT OF STARVATION ON BLOOD PYRUVIC AND LACTIC ACIDS

Numbers in parentheses denote range.

	Pyruvic acid	Lactic acid
	mg. %	*mg. %*
18 hr. starvation	1.3[a] (1.0–1.5)	25 (11–38)
Ad libitum feeding	3.1 (2.4–3.6)	22 (19–28)

[a] Mean value of three rats.

summarized in Table IV. It can be seen that in each experiment, the B_{12}-deprived rats had a significantly higher blood pyruvic acid level than their respective B_{12}-treated litter mates. A correlation of the elevation in blood pyruvic acid and the magnitude of B_{12} depletion was also observed. Analysis by the straight-line regression method indicated that the correlation is significant ($p < 0.01$).

Blood lactic acid level was likewise higher among the deficient animals than the controls with the exception of one control animal in Expt. No. 5. This animal also had the lowest hepatic reserve of B_{12} (110 mμg. g. liver) compared to a group average of 160 mμg. g. liver. Nevertheless, taking all data together, the difference in lactic acid levels among the two groups is significant at approximately the 5% level, by the analysis of variance blocking out the litter effect.

To study the effect of decreased feed consumption as in the case of B_{12} deprivation on blood pyruvic and lactic acid levels, the following experiment was conducted. The animals used were three litter mates grown on a stock diet. Half of them were starved for 48 hr. and the other half were fed *ad libitum*. The results (Table V) indicated that blood pyruvic acid was appreciably lowered after 48 hr. of starvation, whereas blood lactic acid did not appear to be affected.

DISCUSSION

This study indicates clearly that B_{12} deprivation creates an altered concentration in liver DPN and DPNH. The slow response of the disturbed DPN metabolism to vitamin B_{12} repletion suggests that the influence of vitamin B_{12} deficiency on DPN DPNH is probably a secondary one. B_{12} deprivation likewise results in an accumulation of blood pyruvic acid and perhaps lactic acid. These effects of vitamin B_{12} deprivation lend further support on the role of this vitamin in carbohydrate metabolism.

Since pyruvic acid is one of the most reactive and important metabolic intermediates, its accumulation in blood following vitamin B_{12} deprivation may mean impairment in the mechanisms for pyruvate removal. Thus, decreased catabolism of pyruvic acid through oxidative decarboxylation or failure in the conversion of pyruvic acid to oxalacetic acid by carbon dioxide assimilation, and disturbances in the intermediary steps of the tricarboxylic acid cycle could all contribute to pyruvate accumulation. Whether vitamin B_{12} regulates these mechanisms of pyruvate disposal directly or indirectly remains to be elucidated. However, it would not be surprising to have disturbances in the various enzyme systems required for the many pathways of pyruvate removal during the course of B_{12} depletion.

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Effect of Vitamin B_{12} and Folic Acid on Utilization by Rats of Soybean Protein and Amino Acids

YET-OY CHANG AND FUMI YOKOTA[1]

The effect of vitamin B_{12} and folic acid on utilization of soybean protein by rats was investigated. Addition of vitamin B_{12} and folic acid to the soybean diets increased the growth rate as well as the carcass nitrogen retention of rats, which had been depleted of these vitamins. Vitamin B_{12} and folic acid supplementation increased the availability of methionine for rats fed soybean protein. Deficiency in either vitamin B_{12} or folic acid or a combination of these vitamins increased the excretion of urea and decreased the excretion of allantoin in the urine of rats. The ratio of urinary allantoin to urea in rats was the highest when vitamin B_{12} and folic acid were included in the diets.

Many investigators have been searching for an explanation of the effect of vitamin B_{12} in promoting the growth of the animals fed diets containing vegetable protein. Studies have reported that vitamin B_{12} plays a possible role in nucleic acid metabolism (*14*), in the metabolism of methyl group (*15*), and in the formation of enzymes in the liver (*22*).

Vitamin B_{12} has an effect on protein metabolism through its influence upon the synthesis of methionine; thus B_{12} would be expected to improve protein utilization of diets deficient in this amino acid (*7*). Evidences have been recorded regarding the interdependence of vitamin B_{12} and folic acid on methylation reaction as well as in other metabolic processes (*10*, *11*, *13*, *18*). The interrelationship of vitamin B_{12} and folic acid on utilization of protein and amino acids is not thoroughly understood. The object of the present study was to investigate the influence of vitamin B_{12} and folic acid on utilization by rats of soybean protein and amino acids. Four amino acids—lysine, methionine, leucine, and valine—were selected for this investigation. The effect of vitamin B_{12} and folic acid on the excretion of urinary urea and allantoin was also investigated.

Methods

Forty-eight weanling albino rats of the Sprague and Dawley strain were divided into four groups with 12 animals in each group. Four diets were used: Diet 1 contained vitamin B_{12} and folic acid; diet 2 contained neither vitamin B_{12} nor folic acid; diet 3 contained vitamin B_{12} but without folic acid; diet 4 contained folic acid but not vitamin B_{12}. The four diets consisted of the following ingredients (in grams per 100 grams of diet): soybean protein, 18; hydrogenated fat, 8; corn starch, 69; salt mixture (USP 14), 4; and vitamin mix in sucrose, 1. The vitamin mix supplied (in milligrams per kilogram of ration): thiamine, 5; pyridoxine, 5; riboflavin, 10; *p*-aminobenzoic acid, 10; nicotinic acid, 20; Ca pantothenate, 50; D-α-tocopheryl succinate, 75; biotin, 0.3; inositol, 400; choline chloride, 1000; vitamin A, 5000 USP units; and vitamin D, 500 USP units. The vitamin B_{12} supplemented to diets 1 and 3 was 100 μg. per kg. of diet. The folic acid supplemented to diets 1 and 4 was 5.5 mg. per kg. of diet. The soybean protein used in this experiment was a purified protein purchased from Nutritional Biochemical Corp., Cleveland, Ohio.

The animals were initially depleted of their vitamin B_{12} and folic acid reserves by maintenance on a diet devoid of these vitamins but with addition of 0.15% iodinated casein for 2 weeks. Thereafter, the animals were placed on the four experimental diets. Body weight and food consumption of the rats were recorded weekly. At the end of 12 weeks, the animals were placed in metabolic cages; feces and urine were collected for 5 days. The method for collection and treatment of the fecal samples was described in a previous paper (*4*). Fecal amino acids—lysine, methionine, leucine, and valine—were determined microbiologically (*8*). Urine samples were analyzed for allantoin according to the method of Young and Conway (*23*) and for urea according to the method of Engel and Engel (*9*). At the end of 13 weeks, the animals were anesthetized by injection of sodium amytal. The body nitrogen of each rat was determined according to the method of Miller and Bender (*17*). The total carcass nitrogen in 10 weanling rats, killed at the start of the experiment, was determined by the same method. Differences in means were tested, using Duncan's multiple-range test (*21*).

Results and Discussion

Growth and Nitrogen Retention. The growth rates of rats fed the various diets are shown in Table I. The mean weight gain of rats fed diet 1 was the highest. The mean weight gain of the animals singly deficient in vitamin B_{12} or folic acid was higher than those of the doubly deficient rats.

Differences in dietary nutrients result in a difference in body composition. In some cases, weight gain may depend more on a deposition of body fat than an increase of body protein (*1*). In order to assure that increase in body weight indicated an increase in tissue protein, the per cent of carcass nitrogen retained in rats fed the various diets was determined by subtracting the carcass nitrogen at the beginning of the experiment from the carcass nitrogen at the end and dividing this result by

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the total nitrogen consumed. Table I shows that the carcass nitrogen retained in rats of the vitamin B_{12} and folic acid supplemented group and of the singly deficient groups was higher than that in rats of the doubly deficient group. This study indicated that the weight gain of rats under the influence of either B_{12} or folic acid or the combination of the two was a gain in tissue protein rather than in body fat. Also, the effect of vitamin B_{12} and folic acid on carcass nitrogen retention appears to be additive.

Excretion of Urinary Nitrogen Compounds. UREA. The measurement of urea excretion has been employed by many investigators as an index of the quantity and quality of protein ingested as well as the effects of other nutrients on protein utilization (*12*). In recent studies, Kiriyama and Ashida reported that rats fed gluten as a source of protein excreted more urea than rats fed a casein diet (*16*). Schimke found that starvation was associated with an increase in urea excretion, whereas a protein-free diet resulted in a decrease in urea excretion (*20*). In order to ascertain whether vitamin B_{12} and folic acid would exert an effect on protein utilization, the urea excretion of rats fed the various diets was determined (Table II). The ratio of urea nitrogen to total nitrogen of rats fed a diet supplemented with vitamin B_{12} and folic acid is significantly lower than that of rats fed a diet devoid of these two vitamins. There were no significant differences in the ratios of urea nitrogen to total nitrogen in rats among the other groups. Charkey *et al.* have reported that vitamin B_{12} deficiency results in high serum levels of amino acids in the chick (*5*). In this study, the high urea excretion in rats fed a diet deficient in vitamin B_{12} and folic acid may have been related to the high serum levels of amino acids. The inverse relationship of urea excretion with vitamin B_{12} and folic acid supplementation suggests that these vitamins may function in the utilization of free serum amino acids for the synthesis of tissue protein thus reducing renal wastage.

ALLANTOIN. The excretion of urinary allantoin as well as the ratio of allantoin-urea is related to the biological value of dietary protein (*16*). Kiriyama and Ashida found that when rats were fed a casein diet, they exhibited a higher ratio of allantoin-urea compared with those rats fed a wheat gluten diet (*16*). These authors suggested that the allantoin-urea ratio could be used as a measure for the evaluation of the biological value of protein. Following such assumption, the effect of vitamin B_{12} and folic acid on the excretion of allantoin in the urine of rats was determined (Table II). The allantoin excreted by rats in the vitamin B_{12} and folic acid–supplemented group was the highest, while the allantoin concentration was similar in the urine of rats fed the other experimental diets. Since allantoin is the end product of metabolism of the purine base derived from ribonucleic acid, an increase in excretion of allantoin would indicate the increased turnover rate of this nucleic acid.

The ratio of allantoin-urea for rats fed the various diets was calculated (Table II). The vitamin B_{12} and folic acid supplemented rats showed a significantly higher value in allantoin-urea ratio than the singly or doubly deficient rats.

As shown in Table II, vitamin B_{12} and folic acid do

Table I. Effect of Vitamin B_{12} and Folic Acid on the Growth Rate and Nitrogen Retention in Rats[a]

Supplements	Total Gain, Grams	Carcass[b] N, Grams	Carcass N at Start, Grams	Carcass N Retained, Grams	Total N Consumed, Grams	N[c] Retained, %
Vitamin B_{12} and folic acid	159a ± 7.4	4.93	1.56	3.36	21.42	15.70f ± 0.2[d,e]
None	93b ± 6.4	3.94	1.56	2.47	20.53	12.00g ± 0.4
Vitamin B_{12}	133c ± 5.8	4.71	1.56	3.15	23.37	13.47h ± 0.1
Folic acid	128c ± 3.4	4.66	1.56	3.10	22.85	13.55h ± 0.1

[a] Twelve animals in each group.
[b] Carcass nitrogen at the end of 13 weeks.
[c] % N retained = $\frac{\text{carcass N retained}}{\text{total N consumed}} \times 100$.
[d] Mean ± standard error.
[e] Means having the same letter are not significantly different ($P < 0.05$).

Table II. Vitamin B_{12} and Folic Acid on Excretion of Urea and Allantoin in the Urine of Rats[a,b]

Supplements	Urea N, Mg./Day	Total N, Mg./Day	Urea N/Total N, %	Urea, Mg./Day	Allantoin, Mg./Day	Allantoin-Urea, %
Vitamin B_{12} and folic acid	123.6	188.8	65.5a ± 2.8[c,d]	264.9	30.2	11.4c ± 1.0
None	142.2	177.1	80.3b ± 1.0	304.9	25.7	8.4d ± 0.3
Vitamin B_{12}	143.5	179.4	80.0b ± 0.7	307.7	26.1	8.5d ± 0.2
Folic acid	150.0	189.6	79.1b ± 1.4	321.1	26.4	8.2d ± 0.3

[a] Twelve rats per group.
[b] 5-day collection period.
[c] Mean ± standard error.
[d] Means having the same letter are not significantly different ($P < 0.05$).

Table III. Vitamin B_{12} and Folic Acid on the Apparent Availability of Amino Acids for Rats[a,b]

Supplements	Leucine, %	Lysine, %	Methionine, %	Valine, %
Vitamin B_{12} and folic acid	93.16 ± 0.76	94.24 ± 0.32	94.82a ± 0.62[c,d]	94.30 ± 0.41
None	93.60 ± 0.29	94.70 ± 0.39	91.54b ± 0.71	93.97 ± 0.81
Vitamin B_{12}	95.50 ± 0.80	94.17 ± 0.44	93.29a,b ± 0.56	93.39 ± 0.21
Folic acid	92.55 ± 0.74	93.64 ± 0.43	92.56a,b ± 0.97	92.48 ± 0.72

[a] Twelve rats per group. [b] 5-day collection period. [c] Mean ± standard error. [d] Means having the same letter are not significantly different ($P < 0.05$).

not seem to affect the amount of total nitrogen excretion; however, they apparently affect the distribution of the urinary nitrogen compounds such as urea and allantoin. If the excretion of such compounds is related to the nutritive value of dietary protein as has been suggested by previous investigators (*16*), a decrease in excretion of urea or an increase in the allantoin-urea ratio would, therefore, indicate an increase in nutritive value of dietary protein. Apparently, then, the growth-promoting effect through vitamin B_{12} and folic acid supplementation may have resulted from the improved biological value of soybean protein.

Availability of Amino Acids. The availability of lysine, methionine, valine, and leucine for rats as affected by vitamin B_{12} and folic acid was determined (Table III). The apparent availability of amino acids was calculated as follows: From the total amino acid intake subtract the fecal amino acid excreted; then divide this result by the total amino acid intake. Table III shows that the availability of leucine, valine, and lysine for rats of the various dietary groups is practically identical. However, methionine is less available when rats are fed a diet devoid of vitamin B_{12} and folic acid. Previous experiments in the authors' laboratory indicated that vitamin B_{12} and folic acid did not affect the excretion of metabolic fecal amino acids. If the fecal amino acids excreted by rats are derived from exogenous amino acids and metabolic amino acids, the lower availability of methionine for doubly deficient rats would be due solely to the rats' slower release of methionine from soybean protein. This finding is in accord with the in vitro studies of Baliga, Bhagavan, and Rajagopalan (*2*). These authors found that during digestion of soybean protein with trypsin, the release of methionine from raw soybean protein is higher in the presence of vitamin B_{12}. Since methionine is less available only in the absence of vitamin B_{12} and folic acid, folic acid may have a sparing effect along with vitamin B_{12} on the release of methionine from soybean protein. In the doubly deficient rats, their slow growth rate and lower carcass nitrogen retention may be due to lower methionine availability from the soybean.

Reports in the literature concerning the effect of vitamin B_{12} on protein utilization are somewhat contradictory. Baliga and Rajagopalan found that vitamin B_{12} increased the biological value of protein (*3*). Fatterpaker *et al.* found that vitamin B_{12} and folic acid increased the protein content and decreased the fat content in the liver of rats (*10*). In contrast to the above reports, Chow and Barrows (*6*) did not obtain better nitrogen retention with vitamin B_{12} for the rats on a deficient diet. Rupp, Paschkis, and Cantarow (*19*) failed to prove the beneficial effect of vitamin B_{12} on protein utilization. The discrepancies among different workers may be due to the difference in experimental diets or the different methods which were chosen by the investigators for the evaluation of protein utilization. Data obtained in this paper demonstrate clearly that vitamin B_{12} and folic acid influence protein utilization and thereby increase the biological value of protein when excretion of urinary nitrogen compounds, such as urea and allantoin, is used as a measure for protein utilization. The same conclusion can be made when the availability of methionine is used as a measure of protein utilization.

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670. FURTHER STUDIES OF THE EFFECT OF PROCESSING ON SOME VITAMINS OF THE B COMPLEX IN MILK

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This work is a continuation of our earlier studies on the effects of commercial processing methods, as used in Britain, upon the nutritive value of milk(1-13). It had two main objectives: one was to add to the existing knowledge by including measurements of factors which were either unknown at the time of the earlier investigations or for which satisfactory methods of measurement other than biological have only recently been evolved; the other was to study the effects of new methods and recent modifications of existing ones.

Of late the general trend in milk processing has been towards the application of increasingly higher temperatures for short periods of time in various pre-treatments and also towards the use of high temperature in the main treatments themselves. In view of the heat lability of many nutritionally important constituents of milk, the nutritive effects of these new methods obviously demanded study.

The paper deals with seven vitamins of the B complex in seven heat-treated milk products made from one bulk of raw milk.

METHODS

Bulk milk. A bulk of 170 gal. of whole milk predominantly from Shorthorn and Friesian cows was obtained from a commercial dairy within 20 miles of the Dairy Department of Reading University. There it was emptied into an insulated stainless steel tank fitted with a mechanical agitator, and stored overnight at about 5° C. After thorough mixing, samples were withdrawn for processing when the milk was between 24 and 36 hr. old. With the exception of the ultra-high temperature (U.H.T.) sterilization, which was done at the National Institute for Research in Dairying, all treatments were done at the Dairy Department of Reading University on a commercial or pilot-plant scale.

Pasteurized milk (H.T.S.T. process). Eighty gallons of milk were pasteurized at 72° C. (holding for 15 sec.) in a commercial A.P.V. plate-type heat exchanger and the milk was sampled after having been cooled to about 5° C.

Sterilized milk (*in-bottle process*). Four gallons of milk were brought within 10 min. to 71·1° C. and then homogenized at a pressure of 2500 lb./in.^{2} and dispensed into pint bottles which were sealed immediately with 'Crown' corks of the type normally used. The bottles were heated in a steam autoclave for 30 min., some at 107·2° C. and others at 111° C.

Sterilized milk (U.H.T. *process*). Fifty gallons of milk were brought within 18 sec. to 85° C., homogenized in a two-stage homogenizer at 500 and 2000 lb./in.^{2} within about 23 sec. and sterilized by the ultra-high temperature process in an A.P.V. 200 gal./hr. HX plate-type stainless steel commercial plant. The milk was heated in it within 18·5 sec. to 135° C. and held at this temperature for about 2 sec. before cooling to 20° C. One gallon of this processed milk was dispensed into pint bottles, which were then sealed with 'Crown' corks and heated to 107·2° C. for 20 min. The second sterilization was necessary to simulate commercial conditions in which there is still no satisfactory method for aseptic bottling.

Evaporated milk. Fifteen gallons of milk, preheated at 95° C. for 10 min., were evaporated in a small stainless steel vacuum pan at a temperature between 52° and 54° C. to a specific gravity of 1·072 at 49° C. (33·9% milk solids). The milk reached this concentration in about 45 min.; 5·5 g. of sodium citrate were then added and the milk was homogenized at 2000 lb./in.^{2}. The citrate-stabilized, homogenized milk was then filled into standard (2-pint equivalent) cans and sterilized in a steam autoclave at 115° C. for 15 min. The cans were cooled in water immediately on removal from the autoclave.

Sweetened condensed milk. To 15 gal. of milk were added 25½ lb. of cane sugar and the mixture was preheated at 79·5° C. for 15 min. The evaporation was carried out in the same pan and under the same conditions as for the evaporated milk, but the striking point was 1·282 specific gravity at 49° C. (77·2% total solids including 33·8% milk solids). The condensed milk was then cooled to about 32° C. in a tubular condensed milk cooler made with stainless steel tubes and gun-metal end caps and, after standing for about 15 min., was further cooled to 24° C. before being filled into condensed milk cans of a standard size, 1¾ pints equivalent.

Roller-dried milk. Two gallons of milk were heated to 71·1° C. within 10 min. and then dried in a pilot-plant roller drier on twin rollers 8 in. long and 8 in. in diameter heated by steam at 60 lb./in.^{2}.

Spray-dried milk. Three gallons of milk were heated to 87·8° C. and then evaporated under reduced pressure in a glass apparatus at 45° C. in about 45 min. to about 40% milk solids. The concentrated milk, divided into two equal batches, was then spray-dried, in a Niro pilot plant in which milk comes in contact only with stainless steel and which uses a high-speed rotating wheel for atomization. Two different chamber temperatures were used: (*a*) 'low temperature'—inlet 150° C., outlet 75° C.; and (*b*) 'high temperature'—inlet 180° C., outlet 80–90° C. Each batch took 1 hr. to collect. The milk powder was on the average in the hopper half this time at 75° or 90° C. The collecting times were considerably longer than those under commercial conditions.

Sampling. After preparation, the several products were placed in a cold room at 3° C. Samples of each were then taken for the various determinations. The samples for microbiological tests were stored at −30° C. until required. The physico-chemical tests were completed within 48 hr. of sampling, and the samples were not frozen.

Methods of vitamin assay

The following vitamins were measured: thiamine, riboflavin, nicotinic acid, pantothenic acid, biotin, vitamin B_6 and vitamin B_{12}. In general, published procedures were used which are briefly outlined below. Ford, Gregory, Porter & Thompson (14) and Gregory (15) give fuller details.

Chemical methods

Preparation of samples. Products that had been concentrated or dried were reconstituted to the same total milk solids content as the original milk. Sampling for assay was by means of pipettes calibrated for weight delivery of each particular product.

Thiamine was measured fluorimetrically by the thiochrome method of Jansen (16) in a Cohen-type fluorimeter (17), as described by Houston, Kon & Thompson (18). In addition, recovery tests were done with crystalline thiamine added just before the stage of oxidation to thiochrome.

Riboflavin. The methanol method of Emmerie (19) was applied with the omission of the potassium permanganate–hydrogen peroxide step which we have found unnecessary with cow's milk and its products. The fluorescence was measured in the same fluorimeter as for thiochrome, but a yellow filter (Wratten No. 12) was placed between the fluorescent substance and the photocell. The fluorescence was compared with that of a solution of known strength of crystalline riboflavin in 50% aqueous methanol.

Microbiological methods

Nicotinic acid. Weighed samples were heated with 0·2 N-H_2SO_4 for 20 min. in steam at 20 lb./in.^{2} pressure. The extracts were then brought to pH 4·8, diluted with water to known volume, and filtered. After adjusting to pH 6·8 the filtrates were assayed with *Lactobacillus arabinosus* 17–5 in the medium of Roberts & Snell (20), modified by omitting nicotinic acid and including riboflavin.

Biotin. The samples were prepared for test as for nicotinic acid. The same test micro-organism was used, and the same growth medium, modified by omitting biotin and including riboflavin.

Pantothenic acid. Weighed samples were dissolved in water and heated in flowing steam for 20 min. The solutions were cooled, made to pH 4·8 at known volume and filtered. The filtrates were made to pH 6·8 and assayed with *Lb. arabinosus* 17–5 in the medium of Roberts & Snell (20), modified by omitting pantothenic acid and including riboflavin.

Vitamin B_6. Weighed samples were autoclaved with 0·055 N-H_2SO_4 for 5 hr. at 20 lb./in.^{2} steam pressure, as recommended by Rabinowitz & Snell (21). The extracts were then adjusted to pH 4·8 at known volume, filtered and assayed with *Saccharomyces carlsbergensis* 4228 by the method of Atkin, Schultz, Williams & Frey (22) with the following modifications. The medium was supplemented with nicotinic acid and ammonium phosphate, as recommended by Hopkins & Pennington (23); the final liquid content of assay tubes was 5 ml.; the filled tubes were heated in flowing steam for 15 min., cooled in ice before inoculation and brought to 30° C. in a water-bath before transfer to a shaker in an air incubator.

Vitamin B_{12}. Extracts were prepared as recommended by the Analytical Methods Committee, Society for Analytical Chemistry (24), and assayed with *Lb. leichmannii* ATCC 4797 by the method of Skeggs, Nepple, Valentik, Huff & Wright (25), modified as described by Coates, Ford, Harrison, Kon & Porter (26). Some of the results were checked by assays with *Ochromonas malhamensis* (cf. Ford (27); Coates & Ford (28)).

Table 1. *Effect of different processing treatments on some of the vitamins of the B complex in milk*

Description of milk	Product: Total milk solids (g./100 g.)	Product: Fat content (g./100 g.)	Product: Concentration factor (calculated from fat content)	Vitamin content: Thiamine µg./g.	Thiamine: Loss on processing (%)	Riboflavin µg./g.	Riboflavin: Loss on processing (%)	Pantothenic acid µg./g.	Pantothenic acid: Loss on processing (%)	Nicotinic acid µg./g.	Nicotinic acid: Loss on processing (%)	Vitamin B_6 µg./g.	Vitamin B_6: Loss on processing (%)	Biotin µg./g.	Biotin: Loss on processing (%)	Vitamin B_{12} mµg./g.	Vitamin B_{12}: Loss on processing (%)
Raw	12·02	3·30	—	0·46	—	1·6	—	2·6	—	0·72	—	0·22		0·010	< 10	3·2	
Pasteurized (H.T.S.T.)	12·02	3·30	—	0·44	< 10	1·6	0	2·6	< 10	0·71	0	0·20	< 10	0·018	< 10	3·0	< 10
Sterilized																	
In bottle process																	
(a) Before sterilisation	12·00	3·30	—	0·41	< 10	1·6	0	2·6	< 10	0·72	0	0·23	0	0·019	< 10	2·9	< 10
(b) Sterilized at 107·6° C.	12·11	3·40	—	0·30	30–40	1·5	< 10	2·5	< 10	0·75	0	0·23	0	0·018	< 10	< 0·06	90–100
(c) Sterilized at 111° C.	12·07	3·30	—	0·25	40–50	1·5	< 10	2·5	< 10	0·75	0	0·22	0	0·018	< 10	< 0·06	90–100
Ultra-high temperature process																	
(a) U.H.T. alone	12·07	3·30	—	0·44	< 10	1·5	< 10	2·6	< 10	0·73	0	0·23	0	0·017	< 10	2·7	15–20
(b) U.H.T. then in-bottle sterilisation	11·99	3·30	—	0·26	40–50	1·5	< 10	2·3	10–15	0·80	0	0·22	0	0·019	< 10	< 0·06	90–100
Evaporated																	
(a) Before sterilisation	33·90	9·50	2·83	1·2	< 10	4·6	0	6·7	< 10	2·0	0	0·57	< 10	0·050	< 10	2·9	70
(b) After sterilisation	33·54	9·57	2·83	0·62	30–40	4·6	0	7·3	< 10	2·0	0	0·61	0	0·047	10–15	1·0	90
Sweetened condensed																	
(a) Preheated	10·57	3·00	0·88	0·40	< 10	1·6	0	2·4	< 10	0·64	0	0·17	< 10	0·015	< 10	1·0	30
(b) After condensation	33·80	9·50	2·81	1·0	10–20	4·0	< 10	7·5	< 10	2·3	0	0·62	10–15	0·047	10–15	6·4	40
Roller-dried	97·75	26·4	8·36	2·7	30–50	11	10–15	23	< 10	6·0	0	1·8	0	0·14	10–15	21	20
Spray-dried																	
(a) Low temperature	97·43	27·5	8·13	5·7	< 10	12	< 10	24	< 10	6·1	0	1·8	0	0·13	10–15	17	35
(b) High temperature	96·64	26·1	8·26	5·3	10–15	12	< 10	25	< 10	6·1	0	1·6	0	0·13	10–15	16	35

RESULTS

The results are summarized in Table 1. They show that with five of the vitamins—riboflavin, pantothenic acid, nicotinic acid, biotin and vitamin B_6—there was little or no apparent loss during processing.

Thiamine. As was expected (cf. Kon (29)) severe heat treatment resulted in considerable loss of thiamine: a loss of $< 10\%$ during H.T.S.T. pasteurization or spray drying contrasts with losses of 30–40% and 40–50% respectively during in-can sterilization of evaporated milk, and in-bottle sterilization.

Sterilization by the U.H.T. process caused little or no loss. This finding supports that of Bernhard, Gschaedler & Sarasin (30) that 'Uperization' of milk involves relatively little loss of thiamine. 'Uperization' denotes ultra-high temperature short-time pasteurization, and in some essentials resembles the U.H.T. process.

Vitamin B_{12}. Vitamin B_{12} was almost completely destroyed during in-bottle and in-can sterilization. The pre-sterilization treatment of the evaporated milk also caused considerable loss. The preparation of roller- and spray-dried milk entailed about 30% loss, and of sweetened condensed milk about 40%. The first sterilization by the U.H.T. process caused about 15% loss, compared with $< 10\%$ on H.T.S.T. pasteurization.

The values in Table 1 for vitamin B_{12} content were obtained by assay with *Lb. leichmannii*. Assays of several of the products with the protozoan *Ochromonas malhamensis* gave virtually identical results.

Table 2. *Vitamin B_{12} content of proprietary milk products* (mμg./g.)

Product	Vitamin B_{12}
Evaporated full cream, brand A	1·9
Evaporated full cream, brand B	1·0
Evaporated full cream, brand C	1·6
Sweetened condensed full cream, brand D	6·3
Sweetened condensed full cream, brand E	3·0
Sterilized (in-bottle)	0
'National' dried full cream*	22·3 (13–28)

* Six samples, representing different manufacturers' products, supplied for test by Medical Officers of Health at Grimsby, Cardiff, Plymouth, Maidstone, Bradford and Glasgow.

For purposes of comparison, Table 2 shows the vitamin B_{12} content of several commercial and proprietary milk products which, with the exception of the 'National' dried milk, were purchased at local food stores. The values can only be related to a conjectural value for fresh bulk milk, which is likely to approximate fairly closely to 4 mμg./ml.

DISCUSSION

Cow's milk is richer than human milk in vitamins B_6 and B_{12}, and in biotin and pantothenic acid (cf. Gregory (15)). The levels of riboflavin and nicotinic acid are somewhat lower, but on the whole cow's milk has been supposed to provide adequately for the needs of the human infant for these vitamins.

There is, on the other hand, increasing evidence for subtle differences in the chemical forms and associations of certain of the vitamins between milks from different species of animal (cf. for riboflavin, Anonymous (31); for vitamin B_{12}, Gregory & Holdsworth (32), Coates, Gregory, Harrison, Henry, Holdsworth & Kon (33), Gregory, Ford & Kon (34)). Until the metabolism of such vitamin complexes has been more fully investigated it

is perhaps early to assume that they are nutritionally equivalent for the different species.

It may, however, be argued that cow's milk, suitably modified, has been used for many years with apparent success in infant feeding. A more immediate consideration is that of the nutritional implications of the growing use of sterilized liquid milk; for here, in the loss of vitamin B_{12} and the reported inactivation of vitamin B_6, there may well be cause for concern.

Several workers have reported that the biological availability of vitamin B_6 in milk is impaired during the process of heat sterilization (cf. György (35); Tomarelli, Spence & Bernhart (36); Hassinen, Durbin & Bernhart (37)), and in general it appears that the microbiological tests, in particular those made with *Sacch. carlsbergensis* (Hodson (38)), tend to underestimate the extent of this loss on heating. It thus seems that our evidence for the heat stability of vitamin B_6 in milk may be largely illusory, and requires the confirmation of biological tests. These we are now carrying out, together with more refined studies by the methods of chromatography and ionophoresis. There is some inconsistency between different workers' estimates of the heat stability of vitamin B_6 in milk as judged by microbiological methods. It may in part reflect a wide variation in commercial practice in the sterilization of liquid milk in which temperatures from 110° to 121·1° C. are used for periods of from 5 to 30 min. We found that in-bottle sterilization for 120 min. at 115·5° C. caused an apparent loss of 49%, whereas at 110° C. for 30 min. the loss, when measurable, did not exceed 20%. But, doubtless, other factors than the actual process of sterilization are relevant to the reported loss of vitamin B_6 from sterilized and evaporated milk—for example, the prehistory of the milk in relation to aeration and exposure to light; oxygen in the head space; and the period and temperature of storage after sterilization.

Two features of special practical importance emerge from this work: (*a*) that during in-can or in-bottle sterilization serious loss of vitamin B_{12} takes place and, since these sterile milk products have many advantages for infant feeding, this loss may be of nutritional importance; (*b*) that in the recently introduced ultra-high temperature method milk is sterilized without loss of thiamine and with a loss of only about 15% of vitamin B_{12}, but the problem of aseptic bottling remains to be solved before the advantages of this method can be exploited.

The losses of thiamine reported in this paper indicate that some new methods introduced in the last 10 years have increased the adverse effect of processing on the nutritive value.

Further work which is in progress on the factors influencing the heat destruction of vitamin B_6 and vitamin B_{12} will be reported elsewhere.

SUMMARY

1. Pasteurized, sterilized, U.H.T. treated, U.H.T. treated–sterilized, evaporated sweetened condensed, roller- and spray-dried milks were prepared simultaneously from one bulk of raw whole milk.

2. The preparation of any of these products entailed little or no apparent loss of riboflavin, pantothenic acid, nicotinic acid, vitamin B_6 or biotin.

3. Thiamine was destroyed in amounts ranging from less than 10% to 40-50%, increasing with the severity of the heat treatment.

4. Prolonged high temperature used in the sterilization of evaporated milk and of in-bottle sterilized milk caused nearly complete loss of vitamin B_{12}.

5. The new process of sterilization by the U.H.T. treatment alone when it was not followed by the, so far necessary, in-bottle sterilization caused little more loss of the vitamins measured than did H.T.S.T. pasteurization.

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Vitamin B-12 in Amino Acid Metabolism.* (17562)

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McGinnis *et al.*(1) reported that blood non-protein nitrogen content was higher in chicks deprived of animal protein factor (APF) than in normal controls, and suggested that this may have been due to a function of APF in enhancing amino acid utilization. Zucker and Zucker(2) found that non-protein nitrogen, and urea values in rat blood were increased in zoopherin deficiency, and have elsewhere related zoopherin to the animal protein factor. Norris(3) stated that evidence found by his group (not reported in detail) pointed to such a function on the part of APF. The observation of Bird *et al.*(4) that normal chicks have no appreciable requirement for APF after 8 weeks of age may further corroborate the same theory inasmuch as the period during which APF *is* required is the time of most rapid growth, and utilization of amino acids.

It cannot yet be stated with certainty that the effects cited, or those observed in the present work were due entirely to the vitamin B-12 contained in the APF or zoopherin preparations used. There are many indirect indications, however, that the principal activity involved probably was that of vit. B-12. Perhaps the most direct evidence is that obtained by Ott *et al.*,(5) who found that crystalline vit. B-12 exerted APF activity in chicks deprived of animal protein. They concluded that since the crystalline vitamin elicited growth responses comparable to those obtained with crude sources of APF, it is possible that vit. B-12 is identical with or closely related to this factor.

Evidence is presented herewith in more direct support of the theory that vit. B-12 functions in amino acid utilization in chicks. The term 'vitamin B-12' refers hereinafter to an activity provided in the form of the Merck and Co. APF Supplement No. 3.

Experimental. In Exp. 1 mixed single comb White Leghorn chicks from APF-depleted dams were given corn meal for the first 2 days of life, then placed on the experimental diets. Twenty-five chicks were placed in each group, according to a uniform weight distribution pattern. Feed and water were supplied *ad libitum*. The basal diet contained sodium proteinate† 25, corn meal 70, dicalcium phosphate 3, pulverized limestone 1, DL-methionine 0.3, and choline chloride 0.17%. Vitamins and minerals were added, in terms of milligrams per kilogram of diet, as follows: niacinamide 17.5; calcium pantothenate, riboflavin and 2-methyl-1,4-naphthoquinone, each 10; thiamin hydrochloride, pyridoxine hydrochloride, pteroylglutamic acid, p-aminobenzoic acid and alpha tocopherol, each 5; manganese, 27.5; iron, 10; copper, 1; cobalt, 0.1; and sodium chloride, 2500. Vitamin A and D oils were incorporated to provide 6600 I.U. of vit. A and 750 A.O.A.C. units of vit. D per kg of diet. Because certain treatments in this experiment called for the use of iodinated casein, all lots were supplemented to contain 7 mg iodine per kg diet.

In Exp. 2 single comb White Leghorn cockerels from a commercial hatchery were kept on a vit. B-12 depletion diet for 2 weeks; then placed on experimental diets. Twenty-five chicks were placed in each group, according to a uniform weight distribution pattern. The experimental basal diet was as described for Exp. 1 except that iodine supplementation was reduced from 7 to 2.2 mg per kg of diet. The latter iodine level has been found in previous work to be protective against goitre, and experimental treatments did not necessitate balancing at the higher level. The depletion diet was the same as the Exp. 2 experimental basal except that solvent process soybean meal was used at 52.5% and corn meal at 42.5% in place of sodium proteinate at 25% plus corn meal at 70%. To facilitate depletion with respect to vit. B-12,(6) this diet was supplemented with 0.05% iodinated casein (Protamone‡).

Experimental measurements were made of growth, feed consumption, thyroid weight, thyroid histology, and blood contents of: non-protein nitrogen, alpha amino nitrogen, eryth-

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‡ Through the courtesy of Dr. W. R. Graham, Cerophyl Laboratories, Kansas City, Mo.

rocytes, hemoglobin, total amino acids,(7) and 7§ individual amino acids, namely arginine, lysine, methionine, tryptophan, histidine, threonine and valine.

Blood for analysis was collected by ventricular puncture in Exp. 1, and from the carotids after decapitation in Exp. 2. In both cases potassium oxalate was used as the anticoagulant. Individual samples were retained separately until the effectiveness of anticoagulant measures could be verified. Clotted samples and those containing disproportionately large amounts of oxalate were discarded. The remaining samples were pooled according to experimental treatments, and in Exp. 1 according to sex. The pooled samples were stirred gently and subdivided for preparation of both laked(8) and unlaked(9) protein-free filtrates. The filtrates were analyzed for total non-protein nitrogen by the procedure of Koch and McMeekin,(10) and for each of the 7 amino acids by microbiological methods employing *Streptococcus faecalis*, A.T.C.C. 9790.

Of all the various measures taken only growth, feed consumption, non-protein nitrogen and the specific amino acids showed meaningful variations. These are summarized in Table I. The blood contents shown are those obtained by the assay of unlaked protein-free filtrates. Values were obtained by the use of laked filtrates also; but are not reported in detail since this technic provided relatively poor sensitivity to the experimental treatments. The variations encountered in these values show in most instances the same trends as those shown in Table I; thereby lending a degree of further corroboration.

Discussion. It is apparent from Table I that the circulating blood of the birds receiving vit. B-12 contained less non-protein nitrogen, and less of each of the amino acids measured than did the blood from birds deprived of B-12. It also is apparent that the

TABLE I.
Effect of Dietary Vitamin B-12 on Growth, Feed Utilization and Certain Blood Contents.

Exp. No.		Avg init. wt, g	Avg wt gain, g	Avg gain per g feed, g	Blood components—Avg mg % in blood							
					Total NPN	Arginine	Lysine	Methionine	Tryptophan	Histidine	Threonine	Valine
1*	Basal	41	250	0.56	20.7	6.6	5.8	0.77	1.56			
					(5)‡	(4)	(2)	(3)	(4)			
	Basal B-12§	41	266	0.61	18.6	6.6	5.0	0.69	1.43			
					(4)	(6)	(2)	(3)	(6)			
2†	Basal	97	153	0.53	19.0	6.7	7.4	0.57	1.53	1.46	4.7	5.4
					(2)	(3)	(2)	(1)	(3)	(2)	(1)	(1)
	Basal B-12§	96	164	0.60	16.1	5.8	5.3	0.40	1.36	1.11	3.3	4.0
					(1)	(3)	(2)	(1)	(4)	(2)	(1)	(1)

* Duration, 1st 4 weeks of life. Exp. 1 values are averages of both sexes. No sex differences were found in blood contents.
† Duration, 3rd and 4th weeks of life. Exp. 2 was performed with cockerels.
‡ Number of replicate determinations for each value shown in parentheses. Values are averages except those determined only once. Limitations were imposed by exhaustion of materials (protein-free filtrate) for analysis.
§ Merck and Co. APF Supplement No. 3, kindly provided by Dr. D. F. Green. Diet contained the equivalent of 50 μg of vit. B-12 per kg of feed.

7. Danielson, *J. Biol. Chem.*, 1933, v101, 505.
§ Only the first 4 in the case of Exp. 1.
8. Folin and Wu, *J. Biol. Chem.*, 1919, v38, 81.
9. Folin, *J. Biol. Chem.*, 1930, v86, 173.
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birds given B-12 grew more rapidly. Since better growth was obtained at lower blood levels of amino acids, it appears likely that one function of vit. B-12 is to enhance anabolic processes, which remove amino acids from the blood to form fixed tissues. More efficient utilization of blood components leading to lower blood levels could well result in reduction of renal wastage. This in turn should be reflected in greater weight gain per unit of feed; which was found to be the case in both experiments.

The high efficiency of feed utilization observed in these experiments is of interest. Mishler, Carrick and Hauge(11) reported that when a similar ration containing presumably adequate B-12 (as fish solubles) was further supplemented with 0.3% DL-methionine, the methionine gave improved growth and feed utilization. In the present studies the diets were supplemented with 0.3% of DL-methionine, and feed utilization efficiency was very high even in the absence of B-12; although still further enhanced by its presence. A practical type, high energy starter containing animal products (2.5% fish meal, 7.5% meat and bone scrap, 2.5% dried whey) has given at best about 0.45 g gain per g feed. This ration presumably contained ample B-12. Methionine supplementation may have been a requisite condition for the high feed utilization efficiency observed in the present experiments.

The blood methionine levels were unexpectedly low compared to those of the other amino acids, since examination of the amino acid composition of both chicken muscle and of the diets used showed much higher relative contents of methionine than that found in blood. Similar low methionine values (0.106 to 0.317 mg%) were reported relative to other amino acid blood levels in rats.(12) These several findings may reflect some unique relationship of methionine in metabolism, particularly as to the role of vit. B-12 in amino acid utilization.

Summary. Vit. B-12 has been shown to reduce blood levels of non-protein nitrogen and of 7 individual amino acids from the levels found in vit. B-12 deficient chicks.

Chicks given vit. B-12 grew more rapidly and utilized feed more efficiently than B-12 deficient controls, although the latter had higher blood levels of amino acids.

Vit. B-12 appears to function in metabolism by enhancing utilization of circulating amino acids for building fixed tissues.

11. Mishler, D. H., Carrick, C. W., and Hauge, S. M., *Poultry Sci.*, 1948, v27, 263.

12. Wiss, O., *Helv. Chim. Acta*, 1948, v31, 2148.

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SEQUELAE TO THE ADMINISTRATION OF VITAMIN B_{12} TO HUMANS[1]

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THREE FIGURES

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This communication will report some observations on the metabolism and fate of orally and parenterally administered vitamin B_{12}, and on its effect on nitrogen balance in children and on the weight gain of growing children during supplementation of an adequate diet with the vitamin.

GENERAL VITAMIN B_{12} EXPERIMENTS

Metabolism and fate of vitamin B_{12}

Chow and associates (Chow et al., '50) have shown that vitamin B_{12} activity could not be found in the urine of normal individuals or untreated pernicious anemia patients following oral administration of a large dose of 500 µg. Subsequently this lack of B_{12} activity in urine was seen in several individuals receiving as much as 5.0 mg of the vitamin orally (Conley et al., '51). In both types of experimental subjects, striking increase in B_{12} activity in the urine followed a single intramuscular or intravenous injection of 50 to 1000 µg, and the recovery of the injected vitamin was essentially complete. In growing children (see below), we have noted that even

[1] The author wishes to express appreciation to Dr. Francis Schwentker and Dr. B. S. Joslin for their assistance in the several portions of this report. As additional observations are accumulated, these sections will be reported *in extenso* in a series of joint publications.

after 6 months on B_{12}-supplemented diets (daily oral dose, 25 µg) virtually no detectable B_{12} activity was found in the urine. To follow further the fate of vitamin B_{12} after oral administration, we have measured B_{12} activity in the feces of a number of normal individuals before and after administration of a test dose. The results of a typical experiment are given below.

Four males, clinically healthy by the usual criteria, were studied. Preliminary fecal collections were made during two 24-hour periods on each of the subjects. Immediately thereafter, each subject received orally a test dose of 2 mg of crystalline vitamin B_{12}, and feces were collected for another two 24-hour periods. Each fecal sample was homogenized in a Waring Blendor within two hours after defecation and diluted with water to 3 l. Aliquots were autoclaved at 15 pounds pressure for 15 minutes. Upon cooling, they were centrifuged and supernatant fluids were used for assay. Microbiological estimation of B_{12} activity was made on portions of the supernatant liquids, with *L. leichmannii* as the test organism (Skeggs et al., '50). All measurements of B_{12} activity are reported as vitamin B_{12}; it may be likely, but it is not necessarily true, that such assay techniques specifically measure vitamin B_{12}. In preliminary experiments it was found that reproducible results could not be obtained without autoclaving. The data, given in table 1, show a marked increase in vitamin B_{12} in the feces 24 hours following oral administration of doses of 2.0 mg.

These results suggest that more than half of the orally administered B_{12} is either excreted unchanged or modified during passage through the gastrointestinal tract in a way which does not obliterate microbiological activity. These data are in agreement with the findings of Chow and associates ('51), who fed orally a massive dose of a vitamin B_{12} preparation (Chaiet et al., '50) containing radioactive Co^{60} to a group of rats and recovered over 50% of both the microbiological activity and radioactivity in the feces but only a small fraction of 1% in the urine.

Since it seems likely that a requirement for vitamin B_{12} exists in humans and is normally satisfied by the vitamin B_{12} content of foodstuffs, certain alternatives are open for consideration. B_{12} activity in foodstuffs may reside in unknown complexes of the vitamin which are absorbable by most people. There may be a limit to the extent to which material responsible for B_{12} activity, if it is not pure B_{12} itself, is absorbed, and this limit may be close to the level at which it is metabolized to yield inactive urinary excretion products; the balance of an oral dose passes through the gastrointestinal tract either unchanged or metabolized or modified by the bacterial flora to yield only a fraction of the ingested activity in the feces.

TABLE 1

The appearance of an increase in vitamin B_{12} activity in the stool of subjects fed the vitamin by the oral route

HOURS FROM ADMINISTRATION	MICROGRAMS [1] B_{12} FOUND	EXCRETION [2]
		%
—48	30	0
—24	30	0
24	1130	55
48	1330	65

[1] Micrograms of vitamin B_{12} found per 24 hours (average of 4 subjects). No marker was given.

[2] Cumulative per cent of the ingested vitamin excreted (average of 4 subjects; total excretion minus pre-administration excretion).

The distribution of vitamin B_{12} in blood

Earlier results (Conley et al., '51) indicated that about 8 hours after intramuscular administration of a solution containing 1.0 mg of vitamin B_{12} to normal human subjects, blood levels of vitamin B_{12} were essentially nil; most of the injected B_{12} was found in the urine. Samples of citrated blood taken about 15 minutes after injection, however, contained as much as 29 to 34 mμg of vitamin B_{12} per milliliter of blood, while the corresponding plasma samples contained 50 to 80 mμg. These results suggest that vitamin B_{12} does not penetrate into or is not adsorbed onto the red cells within 15 minutes after intravenous administration.

To ascertain whether vitamin B_{12} could penetrate the erythrocytes on prolonged incubation, we added 50 μg of vitamin B_{12} in 0.10 ml of 0.95% NaCl solution to 10.0 ml of freshly defibrinated human blood. The suspension was incubated at 37°C. for three hours under essentially sterile conditions and then centrifuged in an angle centrifuge for 30 minutes. Plasma was carefully removed with a fine capillary. The packed cells (about 5.0 ml) were washed with 1.5 ml of an isotonic saline solution and recentrifuged. Washing with the same amount of liquid was repeated twice more. The cells were then laked with water and diluted to 20 ml. The plasma sample, the combined washings and the laked blood cells were assayed separately for vitamin B_{12}.

TABLE 2

The distribution of vitamin B_{12} in blood (in vitro)

FRACTIONS	B_{12} FOUND	RECOVERY OF B_{12}
	μμ	%
Laked erythrocytes	0.05	0.1
Washings (combined)	4.8	9.6
Plasma	62.5	125.0

The resulting data are given in table 2. Virtually all of the added vitamin was in the plasma, but a small fraction, as would be expected, was found in the washings. Approximately one-tenth of 1% of the vitamin was accounted for in the laked cells. If the vitamin B_{12} were able to penetrate into the erythrocytes under our experimental conditions, the concentration of this vitamin in the plasma should be approximately one-half of that actually found by analysis, since the hematocrit reading was 45% and that in the laked cells

should be many-fold higher. These data demonstrate that vitamin B_{12} does not diffuse into the red cells even upon incubation for three hours at 37°C. There was no evidence of the presence of any enzyme in blood capable of destroying vitamin B_{12}, since the total activity found in the three fractions slightly exceeded the amount of B_{12} added, this difference lying within the experimental error of our routine assay.

Effect of vitamin B_{12} on nitrogen balance in infants

It is generally agreed that vitamin B_{12} is closely associated with the so-called "animal protein factor." Supplementation of a diet containing only vegetable proteins with vitamin B_{12} increased the "feed efficiency" of the diet for pigs (Cunha et al., '50); i.e., less feed was required to produce a given amount of weight gain. This increase in "feed efficiency" does not necessarily indicate an improvement in the utilization of the vegetable proteins in the diet, since these investigators did not measure the retention of nitrogen in the diets with or without supplementation. Chow and Barrows ('50) have shown that in B_{12}-deficient rats the addition of vitamin B_{12} to diets whose sole source of protein was obtained from soybeans failed to enhance the retention of nitrogen; i.e., the per cent of ingested nitrogen retained. These findings suggest, in the light of the observed greater growth of the supplemented animals, an effect of the vitamin on either carbohydrate or fat metabolism, rather than protein metabolism. It has been shown that there is an interrelationship between vitamin B_{12} and dietary fat in mice (Bosshardt et al., '50) and in rats (McCollum and Chow, '50). These observations are, therefore, complementary, and tend to rule out an effect of B_{12} on protein metabolism. It becomes important to extend these findings to humans to ascertain whether a species difference exists and whether vitamin B_{12} supplementation will enhance nitrogen retention in infants fed a diet deficient in this vitamin.

For this phase of our study 4 infants (three males and one female, three to 6 months of age) were offered ad libitum a

basal diet consisting of 100 gm of a soybean preparation and 40 gm of Dextri-maltose No. 1 in water to a final volume of 750 ml. A supplement [2] of a multivitamin emulsion was administered daily. It was thought that feeding these infants soybean meal as the sole source of dietary proteins might deplete them of any reserve of vitamin B_{12}, and therefore they would be more likely to respond to subsequent administration

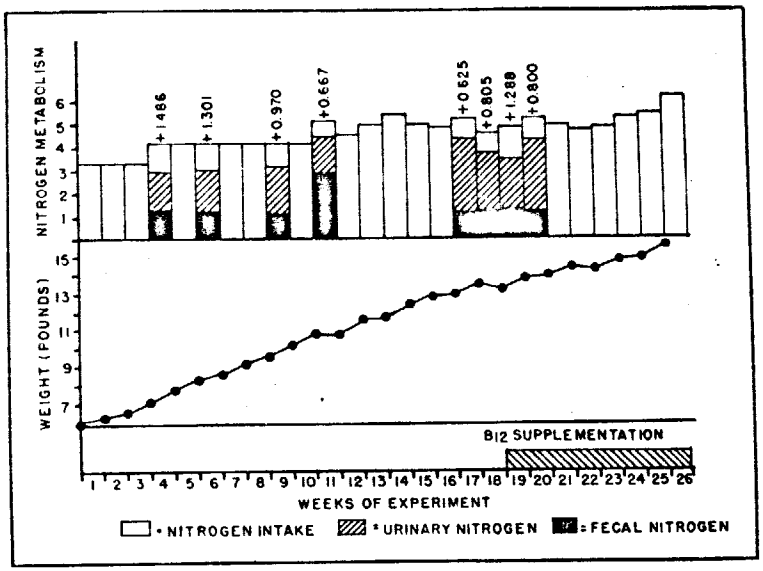

Fig. 1 Nitrogen balance studies on a male infant (GN), 6 weeks old at the start of observation.

of this vitamin. Throughout this time the infants were weighed regularly and nitrogen balance studies were carried out for several weekly periods. At the end of the preliminary period of three months daily supplementation with vitamin B_{12} (10 μg, intramuscularly) was begun, and the nitrogen balance studies continued. Since the values obtained with all subjects were essentially the same, data for one, typical of the group, are shown in figure 1.

[2] These supplements were supplied for this study by Mead Johnson and Hoffmann-LaRoche under the trade names Polyvisol and Vi-Penta, respectively.

The data fail to offer any suggestion that vitamin B_{12} supplementation increases nitrogen retention or the fraction of ingested nitrogen retained. There is no evidence, however, that maintenance of these children on the soybean diet had actually stripped out vitamin B_{12} reserves, or that any deficiency existed prior to supplementation. If the vitamin has any effect on protein utilization, such a role is not shown under the conditions of these experiments. It seems safe to conclude that vitamin B_{12} supplementation, at the levels administered, did not modify protein utilization, as measured by nitrogen balance data, in these subjects.

Growth response of growing children to B_{12} supplementation

It is well-established that the growth rate of a variety of animals, such as rats (Emerson et al., '49), mice (Bosshardt et al., '50), hogs (Neumann et al., '50), and chicks (Ott et al., '48) on a diet deficient in vitamin B_{12} is increased if vitamin B_{12} is added to the diet. Accumulated experience suggests that the enhanced growth rates are reflections of increased food intake ascribable to appetite stimulation.

Whether the vitamin accelerates the growth of children is difficult to decide on the basis of the published reports of Wetzel and his associates ('49) and of Downing ('50). The first group of investigators administered a daily oral dose of 10 μg of vitamin B_{12} to a group of 11 children from a fresh air camp for a period of several weeks. Other resident children, to whom no supplement was given, served as controls. The results led these workers to conclude that this vitamin is effective in promoting the growth response of children with retarded growth.

In the second study (Downing, '50), vitamin B_{12} was given intramuscularly to a group of 25 premature infants. Twenty-three others served as controls. No differences in average total gain before discharge, average daily gain or average time required to reach a weight of 2.5 kg were observed. In both of these investigations, as in the one reported here, it

was not known whether there was any pre-existing deficiency of the vitamin, or lack of it in the diet during the experimental period.

We have been interested in ascertaining whether vitamin B_{12} supplementation can stimulate the weight gain of children receiving a normal diet ad libitum, with substantial supplements of other known vitamins.

It seemed likely, *a priori*, that more striking effects, if they were to be obtained, would be seen in subjects presenting some history of growth retardation as a result of malnutrition or chronic illness. For this reason major emphasis has been placed on accumulation of data from such children but, to round out the picture, some observations have been made on clinically normal children as well. These preliminary observations are reported in this communication in order to bring out the problems peculiar to this type of study and to show the trend of, but not to draw definite conclusions from, our observations on the effects of vitamin B_{12}.

EXPERIMENTAL PROCEDURE FOR THE STUDY WITH CHRONICALLY ILL CHILDREN

Experimental subjects

All the subjects studied in this phase of the program were in a convalescent home. They suffered from chronic illness, such as rheumatic heart disease, simple malnutrition or anemia, minor physical defects, or mental retardation.

Design of study

Before the start of the study, detailed instructions were given to the head nurse in charge with regard to the distribution and care of the prospective subjects. According to the plan, all children, on admission, were numbered consecutively. It was decided in advance that those with even numbers were later to receive vitamin B_{12} supplementation; those with odd numbers would constitute the control group. This method of alternation decided at the time of admission in

which group each child would later fall, without regard to sex, age or disease. Each child was observed for 6 weeks, during which time adjustment to new routines and environment occurred. A majority of these children gained a significant amount of weight during the first two weeks of this period. In most cases the children's weights remained essentially constant during the 4 following weeks. Therefore, 6 weeks after admission the period of experimental observation was begun.

All children were at that time carefully weighed. These weights were considered as the base weights with which the subsequent records were to be compared. All of the subjects received the regular home diet throughout. The children in the control group received no supplement, while those in the experimental group were given an oral supplement of vitamin B_{12}. No attempt was made to give the control group any placebo, since all children in both groups were receiving a variety of medicaments in tablet form as part of their specific treatments. One tablet [3] containing 25 μg of vitamin B_{12} was given daily to children (6 years or older) who were able to swallow it. The younger children in the experimental group, who could not be depended upon to swallow tablets, received daily an equivalent amount of this vitamin in solution.[4] For infants below two years of age, a solution providing 10 μg of crystalline vitamin B_{12} [5] was administered each day by dropper. All children were weighed at regular intervals. The weights of the infants (three years or younger) were recorded every other day to the nearest ounce; children in the older group were weighed weekly to the nearest 4 ounces. The identical scale was used for any one individual during the study. At the time of weighing previous records were not available to the weighers, nor did they know whether any particular subject was receiving the supplement.

[3] Rubramin tablets and Rubramin solutions were supplied by E. R. Squibb and Sons.

[4] See footnote 3.

[5] Crystalline vitamin B_{12} (Cobione) was supplied by Merck and Company.

Dietary regime

All subjects were given food ad libitum. For the infants, care was exercised not to limit their daily milk formula intake. Children in the older group were allowed to have as many helpings of food as they desired, although no persuasion was ever employed. Menus were uniformly well-balanced and diversified as to content. Food consumption data were not accumulated and, as a consequence, it is not possible to estimate reliably the daily caloric intakes or the distribution of calories among protein, carbohydrate and fat. The formula for infants provided milk, Dextri-maltose and water. The vitamin supplements [6] for all children were those regularly used in the home and provided ample amounts of vitamins A, D, B_1, C, and niacin amide but no known source of vitamin B_{12}.

Results

During a study which lasted about one year, a total of 96 subjects were admitted to this home. Out of this number, 24 subjects in the control group and 21 subjects in the vitamin B_{12} group stayed three months or longer following the initial adjustment period of 6 weeks. Records of the weight changes of the others are not included in this report because of insufficient duration of observation. For the 45 children comprising the experimental population, distribution by diseases and by sex and age are given in tables 3 and 4, respectively. In the distribution according to diseases, we find that there were almost equal numbers of children suffering from malnutrition in both groups, but the number of children in the control group with mental retardation or minor physical defects was greater than that in the B_{12} group. Probably a more important difference in the distribution of diseases between the two groups lies in the fact that the B_{12} group contained more children with rheumatic fever or congenital heart disease than the control group. Whether these differences will

[6] See footnote 2, page 328.

eventually change the trends our present data reveal cannot be answered at this time.

From the sex-age distributions (table 4), it would appear that the two groups of males were somewhat balanced against each other. The age distributions of the females are not

TABLE 3

Distribution of diseases

CATEGORY OF INTEREST	RECEIVED B_{12}	CONTROL
Malnutrition	10	11
Mental and physical defects	2	6
Rheumatic heart and congenital heart conditions	8	4
Chronic infection	1	3
Total	21	24

TABLE 4

Distribution by age

AGE IN YEARS	MALE		FEMALE	
	B_{12}	Control	B_{12}	Control
0.5–2	1	2	6	2
2 –4	0	2	0	1
4 –6	1	2	1	3
6 –8	3	3	0	3
8 –10	2	3	3	0
10 –12	2	3	2	0
Total	9	15	12	9

similarly comparable, the experimental group having larger numbers of both very young and older children. The numbers in any corresponding pair of cells are too small to permit valid limited range comparisons to be made. In evaluating the over-all data, the age-sex effects have been considered only to a limited extent.

Grossly, the two groups are comparable with respect to mean weights and their ranges; these values are 42.2 pounds (10 to 83.5) for the control group and 43.0 pounds (11 to 78.5) for the B_{12} group. The mean ages and their standard deviations are roughly comparable, being 69.7 $\pm$ 38.8 months, and 79.1 $\pm$ 44.2 months for the control and B_{12} groups, respectively.

The weight gains of all children in each group, after the initial adjustment period of 6 weeks, are given in figure 2.

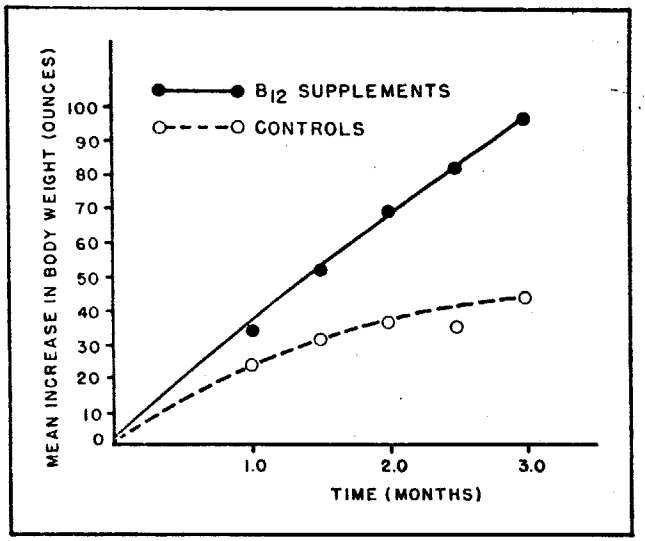

Fig. 2 Average gains of clinically ill children plotted as a function of time, without segregation of the supplemented and control groups with respect to age, sex, or clinical syndrome distribution.

The data demonstrate after three months of vitamin B_{12} therapy the mean gain in body weight in the experimental group was practically twice that in the control group. The difference in mean weight gains began to manifest itself after one month, and consistently increased with time.

At the end of the three months' observation period the difference between the means (53.0 oz.) was statistically very highly significant ($p < 0.001$). The possibility that this re-

sult is a reflection of differences in the age distribution in the two groups is at least partially discounted by the observation that analysis of covariance, using age as the independent variable, does not alter the significance of the difference in the mean weight gains of the two groups, sex difference being ignored.

Moreover, analysis of variance of the accumulated observations at approximately half-month intervals from one month through three months shows an over-all highly statistically significant difference ($p < 0.01$) between the groups, and a highly significant ($p < 0.01$) difference in linear trend with time. Similar analyses of these data following subdivision into two groups according to sex show significant differences ($p < 0.05$) resulting from supplementation and highly significant differences ($p < 0.001$) in linear trend between the supplemented and unsupplemented groups for both males and females. These trend differences support the impression given by figure 2 that the weight changes in the two groups followed markedly diverging trends, which increased with time the separation of weight gains at any given time. Such analyses provide unbiased assessment of the statistical significance of the gross differences, ignoring age, by a method which effectively integrates all of the observations over the period considered. Interpretation from such analyses may be regarded as substantially more meaningful than conclusions drawn from the data at any one point in the course of the study.

Since the subjects here studied represented a variety of clinical conditions, it seems wise to attempt separation by clinical class. Substantial numbers in each of the groups shown in table 3 can be described as malnutrition cases. When data from these individuals are analyzed separately, we have 10 children who received B_{12} and 11 controls. For these groups the weight gains at the end of three months were, respectively, 89.3 and 45.1 oz.; the difference of 44.2 oz. is highly significant ($p < 0.01$). Integrated analysis by the variance technics employed before reveals a highly significant

difference in linear trend ($p < 0.001$) but only a moderately significant difference between groups ($p < 0.05$). This latter may be a reflection of the smaller number of individuals in each group, as well as of the fact that these smaller groups were not well balanced with respect to sex and age distribution.

EXPERIMENTAL PROCEDURE FOR THE STUDY WITH CLINICALLY NORMAL CHILDREN

Experimental subjects

The children studied in this part of our work were resident in a foundling house. The resident population was divided into three age groups (0.5 to 17 months; 18 to 47 months; and 48 to 77 months). At this time, insufficient data on children in the first and third groups are available for statistical analyses. Data on the 18- to 47-month-old group of males only will be reported.

At the time when the study was initiated, there were 24 male children in this home within the age range of 18 to 47 months. The name of each child was recorded on an individual index card. These cards were then shuffled and separated into equal sized control and B_{12}-treated groups by a strictly random procedure.

Design of study

One group received a daily supplement of 25 μg of vitamin B_{12} by addition to milk of 0.5 ml of a solution of 25 mg of the crystalline vitamin [7] in 500 ml of water. The solution was freshly prepared each week.

The control group received similarly a placebo in the form of a solution of 45 mg of vinta certified dye [8] in 2,000 ml of water. The color of the dye solution resembles that of the vitamin solution. These two test solutions were measured in

[7] See footnote 5, page 331.

[8] Vinta dye is a mixture of F. D. and C. certified dyes, supplied by the National Aniline and Chemical Company.

amounts approximately 10 to 15% above the weekly requirements of individual nurses, in two different colored bottles — blue for the vitamin B_{12} solution and brown for the placebo. To minimize further any error in the administration of the solutions, the crib of each infant was also marked with blue or brown corresponding to the color of the bottle from which the supplement was to be administered to its occupant. The solutions were delivered to the nurses weekly.

All children were weighed at a definite hour on a certain day of the week, at which time the weigher was supplied with a mimeographed sheet listing the names of subjects to be weighed, arranged in alphabetical order. At the time of weighing previous records were not available to the weighers, nor were they informed as to whether any given subject was receiving the vitamin or placebo. The records were collected from the weighers on the day of weighing and entered on a master sheet. All weighings on any child were made on the same scale throughout. Weights were measured to the nearest quarter pound.

Results

Population shifts permitted study of only 18 out of the original 24 children over a 24-week period. These children fell into two groups of 9 each which did not differ significantly with respect to age or initial weight. Ages and initial weights for the supplemented and control groups, respectively, were 32.7 ± 8.8 months, 30.6 ± 5.3 pounds; and 32.9 ± 7.8 months, 30.1 ± 4.6 pounds.

The average weight gains of the children in both groups are plotted in figure 3. The mean gain in body weight of the children in the B_{12} group was consistently greater than that of the controls from the 4th week onward. At the end of 24 weeks, the supplemented group showed a weight gain of 58 oz.; the control group, 40 oz. The difference of 18 oz. is statistically significant ($p < 0.05$). Analysis of variance reveals a significant difference in linear trend with time ($p < 0.05$) using biweekly data from the 4th through the 24th

week, but no significant difference in over-all performance between the two groups. It is possible that these findings are fortuitous; nevertheless the consistent trend of the data is strongly suggestive. With an adequate diet, there is no reason to expect very dramatic effects from B_{12} supplementation. If real effects should result from such supplementation, they would be of a small order of magnitude and their statistical evaluation would require additional subjects, or observations over longer periods of time, or both.

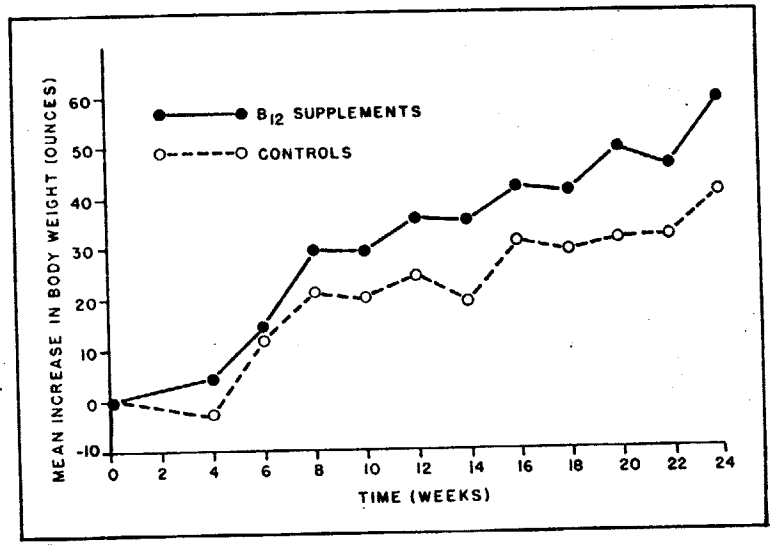

Fig. 3 Average weight gains of supplemented and control groups of clinically healthy male children, 18 to 47 months of age at start of experiment.

Discussion of results

Our data suggest, in general, that vitamin B_{12} may play an important role in the promotion of growth. The greater difference between supplemented and unsupplemented chronically ill children, if it is indeed real and not an artifact, might be conceived as arising from greater deficiency of, or larger requirement for, vitamin B_{12} in such chronically ill children. Our estimates of improved growth rest on greater

weight gains. To the extent that greater weight gain is a criterion of better growth, our findings as to the role of supplementation with vitamin B_{12} in the growth of chronically ill children offer the promise that further study with better balanced groups would clearly point up the benefits to be derived from such supplementation. More data are needed to clarify the situation with respect to normal children.

Our results using chronically ill children are suggestive of a trend of findings similar to that reported by Wetzel and associates ('49). Reconciliation of these findings with those of Downing ('50) is to be sought. It must be recognized, though, that in his study premature infants were used, the period of observation was relatively short, and the published data provide meager bases for evaluating dosage and duration of observations. Furthermore, in the feeding of premature infants it is entirely possible that uncontrollable factors might prevent attainment of the increased food intake necessary to achieve greater weight gains.

The primary objective of the present paper is not to report definitive conclusions on the role of B_{12} as a nutritional factor in the growth of children. We merely wish to report our observations and to point out the problems inherent in the completion of a well-controlled study of this kind. This is timely because of the increasing general interest in the subject, as well as the broader use of oral B_{12} supplements.

In studies such as this many uncontrollable variables inevitably enter the experimental situation. The difficulties encountered in this phase of our study were two-fold. With the chronically ill children the available population was not well balanced with respect to age, sex, or symptomatology, while the data on the well-matched clinically healthy male groups are numerically insufficient. For these reasons the trends apparent in our data are only to be regarded as suggestive; were the experiments less carefully planned it would be impossible to formulate even these tentative ideas.

More important, perhaps, than balancing experimental populations for valid statistical analyses is awareness of the

meagerness of our knowledge of the metabolic role of orally administered vitamin B_{12}. For example, from the published reports on animals and from the data presented in this communication, it is apparent that vitamin B_{12} is not likely to be involved in nitrogen metabolism but may play an important role in carbohydrate or fat metabolism. For this reason, search for the effects of vitamin B_{12} on the growth of any species will result in failure unless the subjects are fed ad libitum. Furthermore, our data demonstrate that the main bulk of orally administered vitamin B_{12} is not absorbed, but excreted in the feces. Questions of dosage and of the route of administration become pertinent. Therefore, the need for studies which might lead to a better understanding of the absorption and utilization of vitamin B_{12} becomes paramount.

In the present investigation we arbitrarily chose a daily oral dose of 25 μg of vitamin B_{12}. There are relatively few data available to serve as guideposts in the choice of dosage in an experiment of this character. In the report by Wetzel et al. ('49), a daily oral dose of 10 μg was employed. Downing ('50) administered the vitamin in smaller doses to premature infants by injection. It has been estimated that the daily oral intake of vitamin B_{12} in what is considered an adequate diet will range from 2 to 5 μg. Our experimental population presented a wide variety of histories from which no conclusions could be drawn as to the possible extent or duration of inadequate dietary vitamin B_{12} intake.

In the treatment of pernicious anemia patients, parenteral administration of 1 μg daily led to desirable clinical response. Oral doses of 5 to 10 μg, together with suitable dietary sources of the intrinsic factor, have also proved satisfactory clinically. In the absence of the intrinsic factor, estimates of the amount of vitamin B_{12} necessary to bring about satisfactory reticulocytosis and clinical improvement have ranged from 25 μg upward to as high as 60 to 100 μg or even 1 mg.

There are no bases for assuming that the dosage necessary for the treatment of pernicious anemia bears any relation to the dosage needed to bring about a growth response of the

type we sought. Some preliminary experiments were carried out with a few children receiving a daily oral dose of 5 μg of the vitamin over a period of two to three months. At this level of supplementation, and on a generally adequate institutional diet, no differences in weight gains were observed during the experimental period. Accordingly, we elected to conduct these experiments using the higher level (25 μg daily of oral vitamin B_{12}), since our purpose was less to establish a dosage level than to seek an effect, even though more critical study, if the effect is established, may subsequently show that this level is excessive.

SUMMARY

1. In confirmation of our previous reports, it was found that oral administration of vitamin B_{12} over a period of several months or in a single massive dose (5.0 mg per adult) did not result in the appearance of vitamin B_{12} activity in the urine. A major portion of the ingested vitamin appeared in the feces. These results indicate poor but apparently adequate absorption of the vitamin in the gastrointestinal tract, even in clinically healthy individuals.

2. When vitamin B_{12} was given intravenously to man, the blood level decreased rapidly and the administered vitamin could be recovered in the urine. Determinations of vitamin B_{12} in plasma and blood failed to yield evidence of penetration into or adsorption onto the erythrocytes within 15 minutes *in vivo*. This was further substantiated in *in vitro* studies, where no appreciable activity could be detected in the red cells obtained from defibrinated human blood previously incubated with vitamin B_{12} for three hours. The vitamin was completely recovered in the plasma.

3. When B_{12} was given parenterally to infants fed on a soybean protein diet, no increase in nitrogen retention was observed.

4. Observations on the effect of vitamin B_{12} on the weight gain of chronically ill and clinically healthy children were made. Data have been presented to show that children of either type gained greater weight than the corresponding controls if offered B_{12} supplementation in a given period. However, due to the heterogeneity of the subjects with chronic illness and the small numbers of the normal children studied, the results are merely considered suggestive of trends which may be confirmed by extended observations under even better controlled conditions.

ACKNOWLEDGMENTS

This work was supported by grants-in-aid from the National Vitamin Foundation, the Squibb Institute for Medical Research, Sharp and Dohme, Inc., and Hoffmann-LaRoche, Inc. The author is also indebted to Merck and Company for the generous supply of crystalline vitamin B_{12}, to E. R. Squibb and Sons for the supply of Rubramin tablets and Rubramin solution, to Hoffmann-LaRoche for the supply of Vi-Penta drops, to Mead Johnson and Company for the supply of milk (Dalactum), Polyvisol, and oleum percomorphum.

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type we sought. Some preliminary experiments were carried out with a few children receiving a daily oral dose of 5 μg of the vitamin over a period of two to three months. At this level of supplementation, and on a generally adequate institutional diet, no differences in weight gains were observed during the experimental period. Accordingly, we elected to conduct these experiments using the higher level (25 μg daily of oral vitamin B_{12}), since our purpose was less to establish a dosage level than to seek an effect, even though more critical study, if the effect is established, may subsequently show that this level is excessive.

SUMMARY

1. In confirmation of our previous reports, it was found that oral administration of vitamin B_{12} over a period of several months or in a single massive dose (5.0 mg per adult) did not result in the appearance of vitamin B_{12} activity in the urine. A major portion of the ingested vitamin appeared in the feces. These results indicate poor but apparently adequate absorption of the vitamin in the gastrointestinal tract, even in clinically healthy individuals.

2. When vitamin B_{12} was given intravenously to man, the blood level decreased rapidly and the administered vitamin could be recovered in the urine. Determinations of vitamin B_{12} in plasma and blood failed to yield evidence of penetration into or adsorption onto the erythrocytes within 15 minutes *in vivo*. This was further substantiated in *in vitro* studies, where no appreciable activity could be detected in the red cells obtained from defibrinated human blood previously incubated with vitamin B_{12} for three hours. The vitamin was completely recovered in the plasma.

3. When B_{12} was given parenterally to infants fed on a soybean protein diet, no increase in nitrogen retention was observed.

4. Observations on the effect of vitamin B_{12} on the weight gain of chronically ill and clinically healthy children were made. Data have been presented to show that children of either type gained greater weight than the corresponding controls if offered B_{12} supplementation in a given period. However, due to the heterogeneity of the subjects with chronic illness and the small numbers of the normal children studied, the results are merely considered suggestive of trends which may be confirmed by extended observations under even better controlled conditions.

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THE INTERRELATIONSHIP BETWEEN VITAMIN B_{12}, STEROIDS AND PROTEIN METABOLISM

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Crystalline vitamin B_{12}[1] has been demonstrated to possess numerous properties of the so-called animal protein factor.[2] From the name animal protein factor it might be inferred that this substance is involved in nitrogen metabolism. Published data from experiments using swine[3] and rabbits[4] indicate that the addition of vitamin B_{12} to basal diets containing corn, etc., affect the amount of dietary nitrogen retained. Chow[5, 6] etc., found that vitamin B_{12} does not affect nitrogen balance in either rats or man even after prolonged priming on a B_{12} deficient diet. These experiments were carried out under conditions where the amount of nitrogen retained is a direct reflection of the efficiency of utilization of dietary nitrogen. They conclude therefore, that vitamin B_{12} does not play a direct role in protein metabolism. Later, Ling[7, 8] et al., demonstrated that the administration of vitamin B_{12} to deficient rats resulted in an increase in the fat content of the carcass but no increase in the per cent of nitrogen retention.

The importance of the pituitary hormones in regulating protein metabolism is well recognized. It has been demonstrated that catabolic hormones such as ACTH or the thyrotropic hormone increase the destruction of tissue proteins. On the other hand, the administration of hypophyseal growth hormone increases nitrogen retention. It is therefore, of interest to ascertain whether vitamin B_{12}-deficient animals can benefit from the injection of the growth hormone.

To this end, 24 male rats born of B_{12}-deficient parents were offered a soybean diet, low in this vitamin. One-third of them was injected with 0.1 mg. of pituitary growth hormone* of high potency daily for two weeks (group A), and another third (group B) received a normal saline solution. The rest was given 1 microgram of vitamin B_{12}. The total gains in body weight at the end of two weeks were: group A, 22 g.; group B, 15 g.; group C, 62 g. Administration of growth hormone did not appreciably affect the growth rate of the deficient rats. As would be expected, the injection of a small amount of vitamin B_{12} rapidly increased the growth rates of the controls, thus indicating the deficiency of our rats. These results therefore, demonstrate that vitamin B_{12} and growth hormone exert their respective effects through different metabolic pathways. While the role of vitamin B_{12} in metabolism or the fate of the injected vitamin in the tissues is by no means fully understood, it is of interest to point out that the injection of vitamin B_{12} to normal or B_{12}-deficient animals results in high concentration of the injected vitamin in adrenals and in the pancreas.[9] The role of vitamin B_{12} in the pancreas for carbohydrate metabolism is suggested by the results of an experiment in which radioactive vitamin B_{12} was injected into a group of normal adult rats. Following injection, the animals were divided into three groups and then offered, respectively, these diets: A, high carbohydrate, low fat; B, adequate fat and carbohydrate diet but deficient in vitamin B_{12}; and C, our stock diet containing a liberal supply of vitamin B_{12}. After three weeks of feeding all the animals were sacrificed and autopsied. Those in group A receiving high carbohydrate-low fat diet showed atrophy of the pancreas and at the same time lost the greatest quantity of the injected radioactive vitamin B_{12} after feeding the test diets

* The author is indebted to Dr. E. Hays of Armour and Company for the generous supply of growth hormone.

(Table I). Although no similar experiments were performed to measure the effects of diet on the adrenals, the high concentration of the radioactive vitamin B_{12} expressed in micrograms per unit weight of organs and the long duration of storage of radioactive vitamin B_{12} likewise indicated to us the possible importance of the adrenals in the metabolism of vitamin B_{12}. The results of a number of experiments showing the interrelationship between vitamin B_{12}, the adrenals and protein metabolism will be described in our discussion this morning.

Table I

Effect of Diets on the Disappearance of Radioactivity in the Tissues of Rats Injected with Radiovitamin B_{12}

Group	Diet	Pancreas		Liver		Kidneys	
		Wt. (gm.)	C. P. M.	Wt. (gm.)	C. P. M.	Wt. (gm.)	C. P. M.
A	HC*	0.460	79	12.0	221	2.2	311
B	HP**	0.883	144	13.6	254	2.4	359
C	SD***	0.910	147	11.9	225	2.3	378

* HC = high carbohydrate-low fat diet.
** HP = high protein-low fat diet.
*** SD = stock diet.

I. *The Effect of Administration of Cortisone on Nitrogen Metabolism*

One of the most important hormones secreted as the result of stimulation of the adrenals is cortisone. It is therefore of interest to study its effect in nitrogen metabolism particularly in enzyme synthesis or destruction. Reports by Silber[10] and our own unpublished data[11] indicate that the subcutaneous injection of cortisone to normal adult rats accelerates the catabolism of tissues with concomitant increases in urinary nitrogen excretion to such an extent as to raise the urea nitrogen: total nitrogen ratio. At the same time the weights of the livers of animals receiving this

hormone were increased over that of the control animals, expressed either as weight per 100 grams body weight of animals or weight of organ per animal. The increase in the liver weight was primarily due to the deposition of glycogen rather than proteins. Administration of this hormone can also bring about a change in enzyme contents of various tissues. For example, the administration of five milligrams of cortisone per day to adult rats weighing about 250 grams increased the arginase activity of the liver but decreased the amylase activity in the same organ. This phenomenon is illustrated in one of our experiments in which two groups of five adult rats were used. In group A, each rat received, daily, 5 milligrams of cortisone, subcutaneously. The second group (B) served as controls. Each animal was given a daily injection of a normal saline solution. During the experimental period, the animals in both groups received equal amounts of stock diet (10 grams per day) to insure that the observations to be made would not reflect differences in caloric intake. After 10 days the animals were sacrificed and the arginase[12] and glutaminase[13] contents of the livers were determined. The results are given in Table II. Treatment with cortisone brought about a marked decrease in body weight, but an increase in both the weight and the total nitrogen of liver. The arginase activity in the livers of the treated animals was higher than that of the controls whether expressed as units per gram or per organ. On the other hand, the injection of cortisone resulted in an increase in glutaminase II activity per gram tissue but not per total organ. The administration of cortisone can thus favor the synthesis of one tissue enzyme over another. The selective destruction of tissue protein as a result of cortisone administration is also illustrated in yet another experiment in which certain specific components of the muscle proteins were measured. In this experiment normal adult rats were injected with five milligrams of

cortisone per day. Three weeks after injection the animals were sacrificed. The muscles obtained from the thigh were immediately frozen and fractionated according to the procedure of Scow.[14] It can be seen from the results (Table III) that administration of cortisone brings about a marked decrease in fraction 3 (which is not precipitable by trichloracetic acid).

Table II

Effect of Cortisone on Liver Enzymes

Group	Body Wt.	Liver Wt.	Arginase		Glutaminase II	
			μ/gm. Tissue	Total μ	μ/gm. Tissue	Total μ
A—Cortisone-treated (4)	311±24*	14.2±.5	25.6±1.2	365±28	209±24	2960±330
B—Control (4)	394±10	11.3±.7	15.7±1.6	176±19	267±42	2970±210

* Standard error.

Table III

Effect of Cortisone on the B_{12}-Binding Fraction of Muscle of Adult Male Rats

	Nitrogen content of each fraction in % of total nitrogen				
	Collagen (1)	Alkalin soluble stroma (2)	Myosin (3)	Water soluble protein (4)	NPN (5)
Control	5	10	30	22	33
Cortisone treated	7	12	20	25	36

What, then, is the relationship between cortisone and vitamin B_{12} metabolism? In our laboratory we have been interested in the fate of vitamin B_{12} following parenteral administration.[15, 16, 17] It was found that as much as 20 per cent of an injected dose of vitamin B_{12} is retained by the muscles of the injected animals. Fractionation of the component which binds with vitamin B_{12} demonstrated that it is likewise fraction 3 which is capable of retaining vitamin B_{12}. It is therefore of interest to ascertain whether

the retention of vitamin B_{12} by intact animals could likewise be effected following cortisone administration. To this end two groups of adult rats were placed in metabolism cages. Each animal in group 1 was injected with 5 milligrams of cortisone daily; those in group 2 received equivalent injections of saline solution. Three weeks after administration all animals were given a test dose of one microgram of vitamin B_{12} tagged with Co^{60}. Urinary specimens collected 24 hours before as well as 24 hours and 48 hours after injection were assayed for radioactivity and for microbiological activity of vitamin B_{12}. Forty-eight hours after the injection of vitamin B_{12} the animals were sacrificed and tissues such as liver, kidneys and 5 grams of muscle from the thigh were removed. Radioactivity in these samples was determined after wet ashing. The results of this typical experiment (Table IV) demonstrate that the vitamin B_{12} activity in the 24 hour-urine specimens of the cortisone-treated animals was approximately twice that of the control animals as measured either by microbiological activity[18] or by radioactivity. The results of tissue analyses demonstrate that in every instance the organs of the treated animals retained markedly less radioactivity than the control ones.

Table IV

Radioactivity in Urine and Tissues After Administration of Labelled Vitamin B_{12}

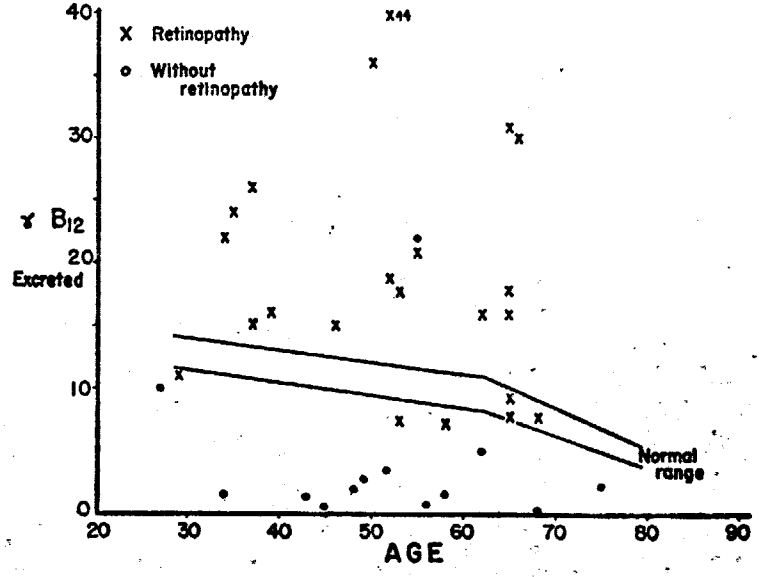
Groups	Treatment	B12 in Urine		mcg. B12 Bound/Gram Tissue		
		R	M	Muscle	Liver	Kidneys
A	Cortisone	0.72	0.77±0.03	0.11	5.7	167
B	Saline	0.44	0.48±0.03	0.18	11.0	244

R = Micrograms of B_{12} as measured by the radioactivity in the 24 hour urine specimen.

M = Micrograms of B_{12} as measured by the microbial activity in the 24 hour urine specimen.

Work at the Wilmer Institute[19] of the Johns Hopkins Hospital has indicated a decreased adrenal function in diabetics without retinopathy and a possible causal relationship of relatively excessive adrenal activity to retinopathy and Kimmel-Stiel Wilson renal lesion. The combination of circumstances described above suggests the hypothesis that the extent of urinary excretion of a test dose of vitamin B_{12} by diabetics might be related to the presence or absence of retinopathy. To test this hypothesis[20] a total of 22 consecutive subjects with retinopathy and 13 without retinopathy, accumulated over a period of four months, were given a vitamin B_{12} tolerance test. There was an uneven distribution with respect to the number of subjects in each of three age groups and a preponderance of females. In spite of this heterogeneity the urinary excretions of B_{12} by all subjects fell into two distinct groups as illustrated in Figure I. In this figure the microbiological

VITAMIN B_{12} TOLERANCE IN DIABETIC SUBJECTS



activity in the urine, expressed as mcg. vitamin B_{12} is plotted against the age of the patient. Over the entire age range, there was uniformly greater excretion by subjects with retinopathy than without save for two exceptions. These were subjects who were originally diagnosed as diabetics without retinopathy, but were found to have (or to have developed) early retinopathic changes on subsequent re-examination. However, in our statistical analyses the original diagnosis were considered. The urinary excretions of clinically healthy subjects were included for comparison. The data show that the diabetics with retinopathy excreted an average of approximately 19 micrograms, whereas the non-diabetic subjects excreted 9.6 micrograms. Statistical analyses indicate a significant difference, not only between the two groups with this disease, but also between either one of them and the healthy subjects. The use of radioactive vitamin B_{12} for injection revealed similar differences in the urinary excretion between the two groups of diabetics (Table V) indicating that the microbiological activity in urine is a true measure of vitamin B_{12}, not interfered with by any inhibitory substance. There seems to be no correlation, however, between the severity of the retinopathy and the B_{12} excretion.

Table V

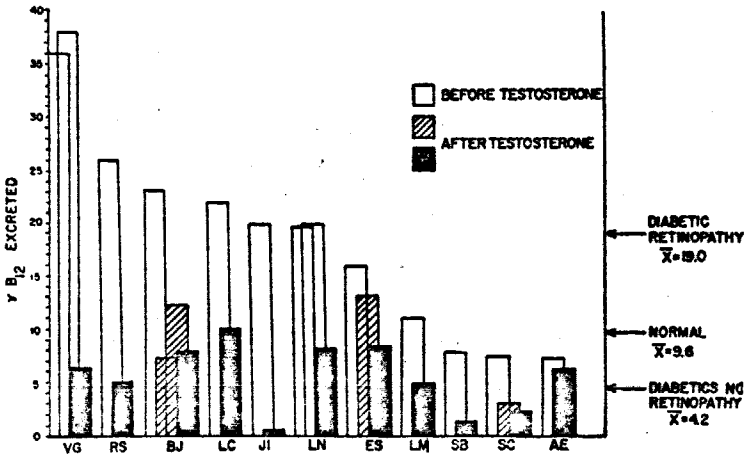
Vitamin B_{12} Excretion and Diabetic Retinopathy

Group	No. of Subjects	B12 by Radioactivity (c. p. m.)	Microbiological activity (mcg.)
With retinopathy	7	4150	17.5
Without retinopathy	4	1100	4.5

Testosterone has been used in the management of diabetics with retinopathy[21]. It was, therefore, of interest to ascertain the effect of this hormone on the excretion of

vitamin B_{12}. To this end, a group of diabetics with retinopathy were first given one, or occasionally two, tests with vitamin B_{12} (50 micrograms) to establish excretion levels before androgen therapy. After intramuscular injections of 100-200 mg. Testosterone Cyclopentylpropionate (Depo Testosterone)* every two to three weeks, these subjects were again given one or sometimes more tests for vitamin B_{12} excretion. Successive tests, when made, were at intervals of at least four weeks. Only after the completion of the microbiological assays for vitamin B_{12} activity in urine was the schedule of testosterone administration compared. The results of such a study are presented in Figure II. It was noted that among the 11 subjects studied there was in every instance a significant decrease in vitamin B_{12} excretion following the testosterone administration although the magnitude of this decrease varied from one individual to another. Whether testosterone will likewise effect the excretion of vitamin B_{12} in non-diabetics or in diabetics without retinopathy remains to be established.

EFFECT OF TESTOSTERONE ON VITAMIN B_{12} TOLERANCE OF DIABETICS WITH RETINOPATHY



* This hormone preparation was kindly supplied by Dr. T. Conger of the Upjohn Company.

If the excessive excretion of administered vitamin B_{12} is related to the adrenal hyperfunction of the diabetics with retinopathy, it might be expected that the subjects with retinopathy would excrete more cortisone in the urine than those without retinopathy or even the non-diabetic individuals. In a preliminary experiment, 18 morning specimens were collected from different diabetics with or without retinopathy and from a group of diabetics with retinopathy previously treated with testosterone. Samples of normal urine were included for comparison. These samples were coded and submitted for analysis for free cortisone-like sterol and for glucuronides. It was found that normal human subjects (5) excreted one milligram or less of free hydrocortisone and about 5 to 10 milligrams of its glucuronide. Diabetics with retinopathy (5) had been found to excrete 10 to 20 times as much free steroid with no increase in the glucuronide fraction. Diabetics without retinopathy as well as those with retinopathy by treating with testosterone excreted amounts similar to the normal individuals. When cortisone or hydrocortisone was administered orally to diabetics with retinopathy who were excreting large amounts of free sterol, the excretion of free sterol was depressed (in 4 out of 5 subjects) to an essentially normal level and the glucuronide excretion was increased. Thus, the diabetics with retinopathy are apparently able to conjugate the sterol when it is administered orally.

In summary it may be stated that data have been presented in this communication to indicate that vitamin B_{12} plays an important role in carbohydrate or fat metabolism and a lesser role in protein metabolism. The fact that the large proportion of the administered radioactive B_{12} is retained by the adrenals when expressed in terms of micrograms of B_{12} per unit weight of tissue organ indicates the

existence of a possible metabolic relationship between vitamin B_{12} and this organ. Biochemical studies on the effect of cortisone, one of the most important hormones produced by the stimulation of the adrenals, on the enzyme systems indicate that administration of this hormone may selectively increase the synthesis of one enzyme over the other. This hypothesis of selectivity is further strengthened by the observation that the administration of cortisone will favor the destruction of one protein component in the whole moiety of muscle proteins. The destruction of this protein as a result of cortisone administration results in inability of the treated animal to retain the parenterally administered vitamin.

Observations were also made that diabetics with retinopathy can retain a much smaller portion of injected vitamin B_{12} than those without retinitis or the normals. These results suggest that such subjects might have hypertrophy of the adrenals. This belief is supported by preliminary observations that a few of the subjects with retinopathy excreted more free cortisone than either normals or diabetics without retinopathy. The existence of a large portion of free steroid in the urine is not due to the inability of these subjects to conjugate glucuronides since the administration of exogenous cortisone or hydrocortisone results in excretion of a large portion of glucuronides. Administration of testosterone to diabetics with retinopathy increases the retention of vitamin B_{12} subsequently administered and at the same time decreases the excretion of free steroid.

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Vitamin B_{12} and the Thyroid in Reproduction of Female Rats[1]

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Olcese et al. ('50), Sure ('51), O'Dell et al. ('51) and Parrott et al. ('60) have demonstrated reproductive failure, including anomalous offspring, in vitamin B_{12}-deficient animals. We have previously reported that in the pregant rat the maternal store of vitamin B_{12} is depleted in favor of that of placenta and fetus, and that human cretins have low serum vitamin B_{12} levels which can be corrected by thyroid therapy (Hellegers et al., '57). Since the lack of thyroid hormone is known to reduce reproductive ability both in man and in experimental animals, it is conceivable that vitamin B_{12} and the thyroid are closely related in the reproductive process. This paper presents exact experimental evidence of the interrelationship between vitamin B_{12} and the thyroid hormone in the reproduction of rats.

EXPERIMENTAL

Animals. Male and femal rats of the McCollum strain from our own colony were used unless otherwise stated. They were 4 to 6 months old, and free from detectable abnormalities. The females had never been bred. In some cases, the thyroidectomized rats were obtained, together with their control animals, from a commercial source.[3]

Breeding. To aid the production of vitamin B_{12} deficiency or inhibition of the vitamin B_{12} action in tissues, the following two agents were used. (a) Inhibitory intrinsic factor (IF)[4] prepared from hog stomach mucosa. Such preparations have been shown to interfere with the absorption of vitamin B_{12} (Williams et al., '57). (b) Vitamin B_{12} antagonist, an anilide of cyanocobalamin.[5]

Determination of vitamin B_{12} activity in plasma and liver. Vitamin B_{12} concentrations in plasma and liver specimens were determined according to the procedure previously described (Yamamoto et al., '57), using Skeggs' medium and *Lactobacillus leichmannii* no. 4797 as the test organism.

RESULTS

Effect of vitamin B_{12} deprivation on reproduction. It is our experience that rats raised and maintained with a vitamin B_{12}-free soybean diet for a long period (three months or longer) lose some reproductive ability. Since the soybean is deficient in other nutrients, such as sulfur-containing amino acids, it can be argued that reproductive failure may not primarily be due to deficiency of vitamin B_{12} alone. In order to minimize the deficiency of dietary protein components, our experimental animals were placed on a good stock diet containing sources of adequate proteins, such as milk. Vitamin B_{12} deficiency was induced by the supplementation of hog intrinsic factor which was shown to decrease vitamin B_{12} absorption, or by the administration of vitamin B_{12} antagonist. Fifty-two young adult female rats were divided into 6 groups (table 1). Five groups were fed our stock diet which has the following components: (in pounds) rolled oats, 18; corn, 20; whole milk powder, 12.5; skim

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[1] The authors wish to acknowledge with thanks a grant-in-aid from the U. S. Public Health Service RG-4916.
[2] Present address: Yamaguchi Medical College, Yamaguchi Prefecture, Ube, Japan.
[3] Thyroidectomized rats obtained from Charles River Breeding Laboratories, North Wilmington, Massachusetts.
[4] Intrinsic factor obtained from Lederle Laboratories, Division of American Cyanamid Company, Pearl River, New York. It was found that the oral administration of this experimental preparation with vitamin B_{12} decreases the gastrointestinal absorption of this vitamin.
[5] Kindly supplied to us by Dr. E. Lester Smith, Glaxo Laboratories, England.

TABLE 1

Relationship between vitamin B_{12} serum levels and reproduction

	Diet and supplement	Body weight	Serum vitamin B_{12}	No. rats mated/no. pregnant	No. rats pregnant/ no. births
		gm	*μg/ml*		
A	Control (stock)	180	0.68	10/10	10/10
B	Stock + inhibitory intrinsic factor[1]	147	0.41	12/3	3/3
C	Stock + inhibitory intrinsic factor + vitamin B_{12}[2]	192	2.0	8/8	8/8
D	Stock + anti-vitamin B_{12}[3]	131	0.71	6/0	0/0
E	Stock + anti-vitamin B_{12} + vitamin B_{12}[2]	188	2.0	6/6	6/6
F	Soybean diet	210	0.40	10/4	4/3

[1] Inhibitory intrinsic factor, 3.5 gm/kg.
[2] Vitamin B_{12} as subcutaneous injection (5 μg three times/week).
[3] Vitamin B_{12}-anilide (50 μg/kg).

milk powder, 12.5; commercial milk protein,[6] 4; NaCl, 0.5; $CaCO_3$, 120; $CuSO_4$, 20; liver powder,[7] 2; ground whole wheat, 30; and ferric citrate, 50 gm. Inhibitory IF was added to the stock diet at the level of 3.5 gm/kg diet for two groups, and vitamin B_{12} antagonist at the level of 50 μg/kg diet for another two groups. One of each of the two groups received in addition 5 μg of vitamin B_{12} by subcutaneous injection three times a week. For comparison, the last group (F) was offered a vitamin B_{12}-free soybean diet. The animals were fed the several diets for 8 weeks and then mated.

The results show that plasma vitamin B_{12} levels of the groups receiving inhibitory IF (B) and soybean diet (F) were considerably lower than that of the control group (A). All of the 10 control rats became pregnant and delivered normally. Fertility in the groups receiving inhibitory IF (B) or soybean diet (F) was low. Only three out of 12 females of group B and 4 out of 10 of group F bore young. None of the 6 rats in group D (vitamin B_{12} antagonist) showed evidence of pregnancy. Administration of vitamin B_{12} to rats treated with inhibitory IF or vitamin B_{12} antagonist (C and E) resulted in fertility and viable young. There were fewer young in the litters of the dams of groups B and F and the incidence of still births in these groups was high. As previously mentioned, these females also had low plasma levels of vitamin B_{12}.

Effect of diet on reproduction and liver vitamin B_{12} concentration of the fetus. Since the preceding experiment demonstrated decreased reproductive capacity of the rats which had lower vitamin B_{12} levels, it was of interest to determine whether the offspring of such animals had correspondingly low tissue vitamin B_{12} concentrations. Accordingly, 48 female rats which had been on the stock diet were randomly divided into three groups. Group A received the soybean diet; group B, the soybean diet supplemented with vitamin B_{12} (1 μg/kg diet), and group C continued to receive the stock diet. Half of the animals in groups A, B and C were mated. At delivery, both the dams and offspring were sacrificed. Vitamin B_{12} concentrations of the livers of the dams and of one young from each litter were determined. These results are tabulated together with the interval between the time of mating and delivery, and number of live young born. Six of the 8 females in group A and all of those in groups B and C delivered young. The average interval between the time of mating and birth of young was considerably greater and the number of live young born was less in group A than in the other two groups (table 2). There was no difference in vitamin B_{12} concentrations in the fetal livers among the three groups and the fetal concentrations were approximately one-half or less than those of the maternal livers.. It is possible that in the fetal liver there is a critical level of vitamin B_{12} below which viable progeny will not develop.

[6] Casal is a commercial preparation of milk protein, Crest Food Company, Ashton, Illinois.
[7] Wilson Company, Chicago.

TABLE 2

Effect of feeding soybean diet on reproduction and vitamin B_{12} content of fetal liver

Diet	No. rats/ no. births	Average interval between mating and delivery (days)	Av. no. live young/ litter	Liver vitamin B_{12} concentration	
				Dam	Young
				$\mu g/gm$	
A Soybean	8/6	33	5.4	71.2 ± 12.9 (81.2 ± 14.7)[1]	36.4 ± 3.5
B Soybean + vitamin B_{12}, 1 $\mu g/kg$	8/8	28	8.8	68.1 ± 16.8 (87.1 ± 13.8)	34.0 ± 0.9
C Stock	8/8	26	10.1	87.1 ± 14.6 (99.7 ± 11.8)	37.8 ± 2.9

[1] Liver vitamin B_{12} levels of the control group fed the same diet, but not mated.

TABLE 3

Interrelationship between thyroid activity and vitamin B_{12} in reproduction

Group	No. rats used/ no. pregnant	No. rats pregnant/ births
A Control	6/6	6/6
B Thyroidectomized	12/0	0/0
C Thyroidectomized + vitamin B_{12}[1]	12/5	5/0
D Thyroidectomized + desiccated thyroid (0.05%) U.S.P.	12/1	1/0
E Thyroidectomized + desiccated thyroid (0.01%) U.S.P.	12/4	4/0
F Thyroidectomized + vitamin B_{12}[1] + desiccated thyroid (0.05%) U.S.P.	12/10	10/10
G Thyroidectomized + vitamin B_{12}[1] + desiccated thyroid (0.01%) U.S.P.	12/12	12/0

[1] Five micrograms vitamin B_{12} by injection, three times/week.

Inadequate amounts of vitamin B_{12} in the diet of the female during pregnancy may be reflected in a reduction of vitamin B_{12} in fetal livers, and a drop in live births.

The effect of vitamin B_{12} and thyroid hormone on reproduction in thyroidectomized rats. The absorption of radioactive vitamin B_{12} is markedly reduced in thyroidectomized rats.[8] Such animals also suffered impaired reproduction. It was therefore, of interest to determine the effect of the administration of vitamin B_{12} and/or thyroid on the reproductive ability of thyroidectomized rats. The following experiment was carried out. Of a large number of female rats, 72 were thyroidectomized, and 6 were sham-operated as controls (group A, table 3); all animals were fed the stock diet. The thyroidectomized rats were divided into 6 groups, 12 per group; one group (B) received no treatment; the second group (C) was given by injection vitamin B_{12}, 5 μg three times weekly; the third group (D) received desiccated thyroid (U.S.P.) in the diet, at the level of 0.05% (50 mg/100 gm diet); the 4th group (E), desiccated thyroid (U.S.P.) at the level of 0.01% (10 mg/100 gm diet). The remaining two groups (F and G) received vitamin B_{12} by injection in addition to the two levels of desiccated thyroid. After 4 weeks, all the animals were mated, and the fertility and live birth rates were determined.

As shown in table 3, fertility of thyroidectomized rats (B) was nil, whereas all 6 of the control group (A) became pregnant and delivered normally. Administration of vitamin B_{12} alone (group C) improved fertility in 5 out of 12 rats, but none of these 5 pregnant animals had living young. Administration of desiccated thyroid U.S.P. at the level of 0.05% (group D) or 0.01% (group E) in the diet re-

[8] Okuda, K., S. L. Steelman and B. F. Chow 1956 Absorption of vitamin B_{12} in hyper- and hypothyroid rats. Federation Proc., 15: 567 (abstract).

TABLE 4

Plasma levels of vitamin B_{12} after thyroidectomy

Group	Months after thyroidectomy	
	1	2
	mμg/ml	
Control	1.03 ± 0.04[1] (5)[2]	0.98 ± 0.06 (4)
Thyroidectomized	0.86 ± 0.05 (5)	0.75 ± 0.07 (4)
P value	< 0.05	< 0.05

[1] Figures are mean ± S.D.
[2] Numbers in parentheses denote number of rats used.

sulted in fertility in one or 4 out of the 12 rats in each group. Administration of both vitamin B_{12} and desiccated thyroid at the level of 0.05% (group F) markedly improved fertility and the birth of live young. Although the rats which received 0.01% of desiccated thyroid in addition to the injections of vitamin B_{12} all became pregnant, none produced viable young. These data demonstrate the interdependence of the supplies of vitamin B_{12} and thyroid extracts.

Males used as breeders for groups B through E in which reduced fertility was observed were later tested for potency. Each was mated with three females. All 15 males appeared to be potent as evidenced by the fact that 43 out of 45 females became pregnant and delivered living young.

These results demonstrate that thyroidectomy reduces reproduction in female rats and the infertility thus produced can be only partially improved by the administration of vitamin B_{12} or desiccated thyroid. Administration of both, however, was more effective in improving reproduction.

Plasma vitamin B_{12} levels after thyroidectomy. Since infertility in thyroidectomized rats was partially corrected by the administration of vitamin B_{12}, and since the plasma level of the vitamin is a good index of the amount available in the body, it was of interest to determine this level in thyroidectomized animals. For this purpose, 10 two-month-old male rats were used. One-half of them were thyroidectomized and the other half served as controls. All animals were fed the stock diet throughout the experimental period. Two months later, the plasma vitamin B_{12} levels of both groups were determined. The results (table 4) show that plasma concentration of vitamin B_{12} decreased significantly after thyroidectomy.

DISCUSSION

Removal of the thyroid gland reduced fertility in female rats. This was partially corrected by the injection of vitamin B_{12}. This suggests that some degree of vitamin B_{12} deficiency develops in thyroidectomized animals. The demonstration of lowered plasma levels of vitamin B_{12} in thyroidectomized rats supports this premise. Since thyroid performs a number of metabolic functions, it is unlikely that reduction in vitamin B_{12} absorption after thyroidectomy is the sole cause of reduction of fertility in athyroidism. Infertility after thyroidectomy was partially but not completely corrected by the administration of desiccated thyroid. Satisfactory reproduction in thyroidectomized rats was obtained only with the combination of vitamin B_{12} and desiccated thyroid at 0.05 % level of the thyroid in the diet. The same combination failed to bring about a normal birth rate when the level of desiccated thyroid was lowered to 0.01%. It is conceivable that vitamin B_{12} and the thyroid hormone are closely related in the reproduction process, and are interdependent in certain biochemical processes during the fetal growth.

SUMMARY

Studies were conducted on female rats on the effects of vitamin B_{12} deprivation and thyroidectomy. The following results were obtained. (a) Female rats, when depleted of vitamin B_{12} either by feeding a vitamin B_{12}-free soybean diet, or by administration of inhibitory intrinsic factor, or by use of vitamin B_{12} antagonist in a complete diet, showed impairment of reproduction. Administration by injection of

vitamin B_{12} corrected both fertility and live birth rate in such animals. (b) Thyroidectomized females did not become pregnant when bred to males of established potency. The infertility due to thyroidectomy was partially corrected by the administration of either vitamin B_{12} or desiccated thyroid. Administration of both vitamin B_{12} and desiccated thyroid fed at a level of 0.05% greatly improved reproduction in thyroidectomized rats. The probable interrelationship between vitamin B_{12} and thyroid hormone in the reproduction of rats has been discussed.

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THE MICROBIOLOGICAL ACTIVITY OF VITAMIN B_{12} IN THE URINE OF NORMAL RATS FOLLOWING THE ORAL AND SUBCUTANEOUS ADMINISTRATION OF THIS VITAMIN [1]

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Recently, Chow and others (Chow et al., '50) demonstrated that the oral administration of vitamin B_{12} to normal subjects or to patients suffering from pernicious anemia did not cause the appearance of the microbiological activity of vitamin B_{12} in the urine. On the other hand, after the intramuscular injection of this crystalline vitamin, at least 50% of the original activity of the injected material was found in the urine within 48 hours. These divergent results with respect to the urinary excretion of this vitamin, together with the relatively ineffectual oral therapy of vitamin B_{12} for the treatment of pernicious anemia patients (Berk et al., '48; Hall et al., '49), raise the question of whether the failure of this substance to appear in urine is peculiar only to man, and hence indicates an inability to utilize the vitamin efficiently as an accessory nutritional factor. It is therefore of interest to ascertain whether oral administration of vitamin B_{12} to another species of animal, such as rats, known to be capable of absorbing and utilizing this vitamin (Emerson, '49), will result in its appearance

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in the urine. To this end, we have fed vitamin B_{12} to a series of normal adult rats both orally and parenterally, and followed the appearance of the microbiological activity of this vitamin in their urine. The results are reported in the present communication.

EXPERIMENTAL PROCEDURE AND RESULTS

The effect of route of administration on the urinary excretion of vitamin B_{12}

In the first experiment 6 normal adult rats weighing between 300 and 350 gm were put into individual metabolism cages and offered for three days a soybean diet supplemented with all known vitamins except vitamin B_{12}. Beginning on the 4th day, one 48-hour collection of urine was made. This specimen was taken as a measure of the basal excretion. On the 6th day, one-half of the prepared animals (group A) were given a vitamin B_{12} solution [3] subcutaneously and the remaining half (group B) received it orally. Fifteen milliliters of an isotonic saline solution were also given by injection daily in order to increase the volume of urine excreted and thereby minimize the error due to the volume of urine retained in the bladders of the animals. Collection of urine was continued for 5 additional days.

Five days after the administration of vitamin B_{12} the route of feeding this vitamin was reversed; thus, the group (A) which previously received vitamin B_{12} by the subcutaneous route now received it orally and the group (B) which had received the vitamin orally now received it subcutaneously. Urine was again collected for two additional days. The vitamin B_{12} activity in each specimen of urine was determined with *Lactobacillus leichmannii* 4797 (Skeggs et al., '50).

The results of the first experiment, shown in table 1, demonstrate that the first group (A) of animals excreted approximately three-fourths of the vitamin B_{12} injected parenterally, whereas the vitamin B_{12} content in the urine of the rats in

[3] Squibb Rubramin concentrate.

group B 48 hours after oral administration was not higher than that collected during the basal period for the same time. However, it should be pointed out that the basal excretion for this group of animals was unusually high. When the route of administration was reversed, we again observed that the major portion of the vitamin B_{12} administered subcutaneously appeared in the urine of the rats of the second group (B), and that there was only a small amount of the vitamin in the urine of the rats receiving B_{12} orally. The appearance of some microbiological activity in the urine of these rats may have been due to the contamination of vitamin B_{12} by the stools. Hence, attempts were made to minimize such an error in our second experiment.

This was achieved by replacement of the soybean diet with a 40% sucrose solution and by the removal of fecal matter within one-half hour after defecation during the working day. It was found that with these precautions the variation in the microbiological activity in the urine specimens of the individual rats collected during the two basal periods was much less than that obtained in the first experiment. We believe that this constancy in the basal values can be attributed to the efforts to minimize the contamination of vitamin B_{12} by the stools. The assay results (see table 1) on the specimens of urine obtained after administration of B_{12} demonstrated again that the vitamin B_{12} given by the parenteral route was excreted regardless of the order of administration. On the other hand, following oral administration of as much as 1.0 mg per rat there appeared in the urine a significant quantity of vitamin B_{12} activity, which amounted to 10 to 50 times the basal excretion and to approximately 1% of the administered vitamin. Since there was a very minute amount of fecal matter in the cage, the major portion of the activity in the urine specimens was probably the result of excretion rather than of contamination by stools or of the production of B_{12} by microorganisms present in the urine.

The data further demonstrate that vitamin B_{12} activity continued to be present in the urine as long as 48 to 72 hours after

TABLE 1

The appearance of vitamin B_{12} activity in the urine of normal adult rats after oral or subcutaneous administration

GROUP A				GROUP B			
Route	Hours from administration	Micrograms excreted	Total excretion [1]	Route	Hours from administration	Micrograms excreted	Total excretion [1]
Experiment 1							
	−48	0.09	0		−48	0.30	0
Subcutaneous (30 μg)	0			Oral (30 μg)	0		
	7	21.0	70		24	0.28	0
	24	1.6	75		48	0.19	0
	48	0.31	76		120	0.34	0
	120	0.51	78	Subcutaneous (30 μg)	0	0	0
Oral (30 μg)	0	0	0				
	24	0.40	1		7	20	67
	48				24	0.25	67
					48	0.18	67
Experiment 2							
	−48	0.07			−48	0.11	
	−24	0.09			−24	0.09	
Subcutaneous (5 μg)	0			Oral (1,000 μg)	0		
					8	0.60	0.06
	8	3.2	64		24	1.47	0.2
	24	0.16	66		48	5.2	0.7
	48	0.11	67		72	1.0	0.8
	72	0.13	68		96	0.28	0.9
	96	0.17	68	Subcutaneous (5 μg)	0		
Oral (1,000 μg)	0						
	8	0.65	0.06		8	3.6	72
	24	0.63	0.1		24	0.24	76
	48	1.8	0.3		48	0.24	78
	72	0.45	0.35		72	0.20	80
	96	0.18	0.36		96	0.15	80
	120	0.18	0.37				

[1] Expressed as per cent of the total vitamin administered.

oral administration, while most of the injected vitamin B_{12} was excreted in 24 hours or less. However, this difference in the rate of excretion may be partially accounted for by the difference in the dosages administered. The pertinent result of this experiment is that the oral administration of as much as 1,000 µg caused the appearance in the urine of only a very small fraction of the microbiological activity of the vitamin B_{12}. It is therefore of interest to ascertain the level of oral intake which must be given in order to cause the appearance of this activity to a marked degree.

TABLE 2

The appearance of the microbiological activity[1] of vitamin B_{12} in the urine of rats following oral administration at different levels

HOURS FROM ADMINISTRATION	TOTAL DOSAGE ADMINISTERED, IN MICROGRAMS					
	10	30	90	270	1000	10^2
	Micrograms of vitamin B_{12} activity in urine					
−48	0.09	0.04	0.14	0.13	0.11	0.11
−24	0.05	0.07	0.04	0.04	0.04	0.06
0	...	...	...	...	...	...
8	0.03	0.03	0.06	0.18	3.2–4.3–5.7	8.0
24	0.03	0.04	0.05	0.65	0.4–10.0–0.7	1.4
48	0.08	0.10	0.07	0.50	0.47–10.5–4.5	0.10
72	0.04	0.12	0.07	0.12	0.35–4.43–8.50	0.04
96	0.08	0.08	0.04	0.05	0.10–0.14–0.10	0.05

[1] All activity results shown are expressed in terms of micrograms of crystalline vitamin B_{12}.

[2] B_{12} was administered subcutaneously.

Level of oral intake of vitamin B_{12} and its urinary excretion

Eighteen normal adult rats weighing 300 to 350 gm were divided into 6 groups of three animals each. The first 5 groups were to receive the following doses of vitamin B_{12} orally: 10 µg, 30 µg, 90 µg, 270 µg, and 1,000 µg per rat; and the 6th group, 10 µg subcutaneously. Like the animals in the second experiment, these rats received a 40% sucrose solution ad libitum. After the collection for two 24 hour periods of the

basal urinary excretion, the specified amounts of the vitamin were given to the respective groups. Collections of urine were made at the following intervals: 8 hours, 24 hours, 48 hours, 72 hours, and 96 hours after administration. The results of the determination of the microbiological activity in the urine are given in table 2.

The data demonstrate that there was no significant increase of the microbiological activity in all the urine specimens from the rats receiving 90 µg or less of B_{12} by the oral route over that in the basal excretion. Hence, it may be concluded that no appreciable amount of the fed material appeared in the urine. On the other hand, when the oral dose was increased to 270 µg, a small amount of vitamin B_{12} was excreted. The excretion of vitamin B_{12} increased as the oral dosage was further increased to 1,000 µg. The results of the microbiological assay of the urine specimens of three individual rats receiving 1.0 mg B_{12} are given because of the large variations among the rats used. In spite of this variation, there is no doubt that this group of animals excreted a considerable amount of vitamin B_{12}. The delayed excretion after the oral administration, which was pointed out in our second experiment, was again noted in this series of rats. As would be expected, the administration of 10 µg of vitamin B_{12} subcutaneously caused the total excretion of the injected material within 24 hours.

Effect of repeated oral administration of vitamin B_{12} on urinary excretion

An experiment was designed to ascertain the effect of repeated oral administration of even higher doses of vitamin B_{12} on the appearance of microbiological activity in the urine. To this end 8 normal rats were offered a soybean diet and then a 40% sucrose solution. The collections made during the first two 24-hour periods were taken as the basal excretion. The animals were then divided into three groups. Group A (4 rats) were given 2 mg of B_{12} orally and another 2 mg 4 hours later; thus the total dosage was 4 mg. Group B (two rats) were

given the identical amount of this vitamin, at the same intervals, by the subcutaneous route. Group C (two rats), which received two injections of a normal saline solution during the same time intervals, served as a control.

The results of microbiological assay (table 3) showed that animals receiving the massive doses of vitamin B_{12} solutions by the subcutaneous route excreted the entire portion of the vitamin in the urine within 24 hours after the first injection, although the microbiological activity continued to appear in the urine, in amounts greatly exceeding the basal value but

TABLE 3

The appearance of vitamin B_{12} activity[1] in the urine of normal adult rats after oral or subcutaneous administration of two massive doses (1.0 mg per rat) at close time intervals

HOURS FROM ADMINISTRATION	GROUP A (Oral route)	GROUP B (Subcutaneous route)	CONTROL GROUP (No B_{12})
	Micrograms of vitamin B_{12} activity in urine		
—48	0.04	0.03	0.07
—24	0.05	0.03	0.04
0			
24	65.0	4,050.	0.03
48	71.0	109.0	0.08
120	28.0	72.0	0.38

[1] All activity results shown are expressed in terms of micrograms of crystalline vitamin B_{12}.

equivalent to only a small fraction of the administered dose, even after 120 hours. The animals which received the massive doses orally excreted only a small fraction (about 4%) of the administered vitamin. Assay of the microbiological activity in the urine of rats in the C group demonstrated that the basal excretion of the animals on the sugar diet was essentially constant except for the last period, when it was unusually high. We believe this to have been due to fecal contamination.

These results therefore demonstrate that in spite of the oral administration of two massive doses of vitamin B_{12} at intervals of 4 hours, only a small amount of the microbiological activity appeared in the urine; whereas subcutaneous injections of the same amount of this vitamin caused the appearance in the urine of almost the entire amount of the administered vitamin.

DISCUSSION

It is well known that the oral administration of vitamin B_{12} for the treatment of pernicious anemia patients is less effective than the parenteral administration unless the oral administration of this vitamin is accompanied by the gastric juice obtained from normal individuals, or unless the dosage is increased many-fold. These facts may be taken to indicate that only a small amount of the vitamin B_{12} given orally is absorbed. The lack of appearance of vitamin B_{12} activity in the urine of normal subjects or of pernicious anemia patients can also be explained on the basis of poor absorption. It is therefore of interest to ascertain whether similar results can be obtained in another species of animal, such as rats, where it has been demonstrated that the administration of vitamin B_{12} by the oral route is effective for the promotion of growth, reproduction, and other important physiological functions. The results presented in this paper demonstrate that the oral administration of crystalline vitamin B_{12} to normal adult rats, as to man, did not cause the appearance of its microbiological activity in urine if the dosages were no higher than 90 µg per 300 gm of body weight. This dosage is equivalent to about 21 mg for a 70 kg man. The biggest dosage of crystalline vitamin B_{12} which we have so far administered to man is 5.0 mg. At this level, there was no detectable B_{12} activity in the urine.

When the dosage was increased to 270 µg per rat of the same body weight, some microbiological activity appeared in the urine. Further increase of the oral intake by 10- or 15-fold, either as a single dose or as repeated dosages, failed to increase materially the microbiological activity or to prolong greatly the presence of the activity in the urine.

The results presented in this paper therefore demonstrate that the rat, like man, does not excrete appreciable quantities

of vitamin B_{12} in the urine, when it is given orally, unless the dosages are very high. Since feeding vitamin B_{12} to rats, by mouth, in dosages much smaller than those used in our experiment can stimulate growth, reproduction, and other important functions, the absence of the microbiological activity in the urine cannot by itself be taken to indicate an inability to absorb the vitamin.

SUMMARY AND CONCLUSION

Vitamin B_{12} was given to a series of rats both by the oral and subcutaneous routes. Vitamin B_{12} activity was found to be absent in the urine of animals receiving B_{12} orally until a dosage of at least 270 μg/300 gm rat was reached. At this or a higher level, only a small fraction of the administered vitamin appeared in the urine. The administration of crystalline vitamin B_{12} to rats by the subcutaneous route caused the appearance of the microbiological activity of this vitamin in the urine in amounts essentially equal to the amount injected. Since it has been demonstrated that rats can absorb and utilize vitamin B_{12} for normal physiological functions, and since this species of animal, like man, cannot excrete this vitamin following oral administration, the absence of vitamin B_{12} activity in human urine cannot be taken as evidence that the vitamin is not absorbed or utilized by man.

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Reports of Cases

NUTRITIONAL VITAMIN-B_{12} DEFICIENCY

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There have been several reports of the effects of vegetarianism (abstinence from meat) and veganism (abstinence from all animal products) on vitamin-B_{12} serum levels and on vitamin-B_{12} deficiency states from countries other than Australia (Banerjee and Chatterjee, 1960; Hardinge and Stare, 1954; Badenoch, 1952; Wokes *et alii*, 1955; Harrison *et alii*, 1956). To our knowledge, there have been no previously reported instances of nutritional vitamin-B_{12} deficiency in Australia. Two cases of this condition recently investigated at Royal Perth Hospital are therefore presented.

Methods

The serum vitamin-B_{12} concentrations were estimated by the microbiological assay method of Hutner, Bach and Ross (1956). The serum folic acid levels were estimated by the microbiological assay method of Herbert (1961). The formiminoglutamic acid urinary excretion was estimated by the method of Davis and Onesti (1960). The radioactive vitamin-B_{12} absorption studies were performed by the urinary excretion technique described by Pitney and Stokes (1958). The xylose absorption test (Case I) was performed by the method described by Benson *et alii* (1957).

Case I

A woman, aged 75 years, was referred to the Royal Perth Hospital for investigation of anæmia of recent onset. Her complaints were of weakness of some months' duration, ankle swelling, chest pain on exertion and occasional right upper abdominal pain. There was no significant past or family history. For 15 years prior to her admission to hospital, the patient had refrained from eating meat in any form because of religious convictions. For one year she had drunk no fresh milk, although she occasionally used small amounts of condensed milk. She had for many years eaten very few eggs, and for some months prior to her admission to hospital had refrained from them completely. Also, for many years she had eaten no butter, but instead used vegetable margarine. Over recent months she had abstained from potatoes. Nevertheless, to direct questioning she replied that she had "a good diet", and analysis of her eating habits showed that she had a daily intake of 2400 Calories; her daily intake of first-class protein was nil, of second-class protein 41 grammes, of carbohydrate 284 grammes and of fat 130 grammes. She did not consume alcohol. Physical examination showed the patient to be very pale and in mild congestive cardiac failure. There were no abnormal neurological signs. The tongue was normal.

On her admission to hospital, the hæmoglobin value was 6·0 grammes per 100 ml., the white cells numbered 3700 per cubic millimetre, and the red cells showed poikilocytosis, polychromasia and a moderate anisocytosis; many macrocytes were noted. The bone marrow was very hyperplastic, and erythropoiesis was magaloblastic in type. Occasional giant metamyelocytes were noted. The vitamin-B_{12} concentration of the serum was less than 40 $\mu\mu$g per millilitre of total vitamin B_{12} and less than 20 $\mu\mu$g per millilitre of free vitamin B_{12}. Histamine-fast achlorhydria was demonstrated by a histamine test meal. A barium-meal and follow-through X-ray examination showed the

stomach to be hypotonic and the mucosal folds to be ironed out by the barium. The changes were interpreted as being typical of gastric atrophy (Joske and Vaughan, 1962). Gastric biopsy was performed, and the specimen was reported as showing infiltration of the lamina propria with polymorphs, leucocytes and plasma cells; there was mild glandular atrophy, but plentiful specialized secretory cells were still present. The level of formiminoglutamic acid excreted in the urine collected between three and eight hours after an oral dose of 15 grammes of histidine was zero. The concentration of folic acid in the serum collected on the day of the patient's admission to hospital was 8·1 mμg per millilitre (normal range, 2·8 to 18·3 mμg per millilitre). Radioactive vitamin-B_{12} absorption studies were performed on two occasions. On the first occasion, after an oral dose of 0·6 μg of ^{58}Co labelled vitamin B_{12} given without any added intrinsic factor, 35·3% of the administered radioactivity was recovered from the urine passed over the following 48 hours. When the same investigation was repeated, 19·3% of the administered radioactivity was recovered from the 48 hour urine samples (normal value, greater than 16%). The serum bilirubin content was less than 0·5 mg. per 100 ml., the blood urea content was 25 mg. per 100 ml. and the plasma carotene content was 99 μg per 100 ml. (normal value greater than 70 μg per 100 ml.). Xylose absorption was normal.

At the time of the patient's admission to hospital, the significance of her dietary history was not appreciated. On the basis of the clinical picture, the peripheral blood examination and bone-marrow biopsy, a tentative diagnosis of Addisonian pernicious anæmia was made. Later, the findings of a lowered serum vitamin-B_{12} level, the presence of gastric atrophy suggested radiologically and the presence of histamine-fast achlorhydria appeared to support the diagnosis strongly. Treatment, consisting of 100 μg of vitamin B_{12} given by intramuscular injection twice a week, was commenced on the seventh day after the patient's admission to hospital. The subsequent rise in the hæmoglobin value and the reticulocyte response are shown in Figure 1 and Table I. Marked clinical improvement paralleled the rise in the hæmoglobin value. The finding of a maximum reticulocyte count occurring as early as the fourth day after the first injection of vitamin B_{12} was surprising, and raised doubts as to the validity of the diagnosis of pernicious anæmia. It was thought at the time that the patient might, in fact, be suffering from a nutritional (folic-acid deficient) megaloblastic anæmia which was responding to ward diet. However, the lack of formiminoglutamic acid excretion after a histidine load did not support this suggestion. Later, results of serum vitamin-B_{12} and serum folic-acid estimations came to hand; the radio-active labelled vitamin-B_{12} absorption studies were performed, and the probable true nature of the ætiology of the megaloblastic anæmia was appreciated.

In retrospect, the patient states that during her stay in hospital, she ate all the ward diet except the meat presented to her. This included milk, eggs and butter. It is now believed that it was the vitamin B_{12} contained in the ward diet which allowed the earlier than expected reticulocyte response.

Case II

A man, aged 76 years, presented with a six-weeks history of progressive weakness of the lower limbs. This had first appeared fairly quickly over a period of a few days, and then had slowly but steadily worsened until, on his admission to hospital, he required the aid of a stick in walking. There were no other relevant symptoms and no relevant past or family history. For approximately 30 years the patient had been a strict vegetarian, and for 18 years he had been a complete vegan. His diet was estimated to contain a daily intake of approximately 245 grammes of carbohydrate, 47 grammes of fat, 29 grammes of second-class protein and no first-class protein, giving a total of about 1560 Calories.

Examination showed the patient to be a small, lightly built, elderly man, with a mildly spastic gait. He was mentally alert, but had a rather poor memory for recent events. In the lower limbs there was moderate weakness

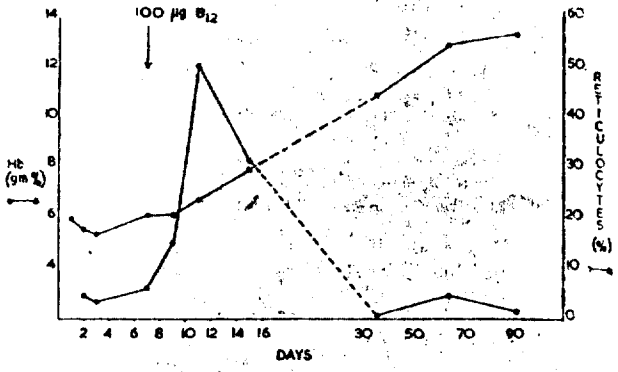

FIGURE I

of all muscle groups, with no wasting, but with increased muscle tone, hyperreflexia and extensor plantar responses. There was loss of position sense in the toes with generalized impairment of vibration sense, which was considered to be within normal limits for a man of his age. The only other significant abnormality found on physical examination was tenderness over the spinous processes

TABLE I

Day	Hæmoglobin Value (Grammes per 100 ml.)	Reticulocytes (Percentage)
1	6·0	—
2	5·6	4·8
3	5·4	3·4
7	6·1	6·1
9	6·1	15·0
11	6·7	50·3
15	7·9	31·2
36	10·9	0·6
64	12·9	4·5
91	13·8	1·3

of the third, fourth and fifth thoracic vertebræ. Blood examination showed a hæmoglobin value of 14·8 grammes per 100 ml.; 3·8% of the red cells were reticulocytes; the leucocytes numbered 6000 per cubic millimetre and the platelets 270,000 per cubic millimetre. A peripheral blood film and a sternal marrow biopsy both gave normal findings. Serum collected on the day after the patient's admission to hospital had a total vitamin-B_{12} content of 45 μμg per millilitre and a free vitamin-B_{12} content of less than 20 μμg per millilitre. The serum folic acid level was 28·4 mμg per millilitre (normal range, 2·8 to 18·3 mμg per millilitre). No formiminoglutamic acid was found in the urine collected between three and eight hours after a loading dose of 15 grammes of histidine. After an oral dose of 0·6 μg of radioactive labelled vitamin B_{12} given without added intrinsic factor, 27·6% of the administered radioactivity was recovered from the urine passed over the following 48 hours (normal value, greater than 16%). A histamine test meal showed elevated gastric acid secretion. X-ray examination with a barium meal was reported as showing normal findings, with well-marked rugæ along the greater curvature of the stomach. A gastric biopsy showed the gastric mucosa to be reduced in depth, with moderate glandular atrophy, but with parietal cells still readily found; there was evidence of active chronic gastritis, the lamina propria containing lymphoid nodules and being diffusely infiltrated by lymphocytes, plasma cells and polymorphs. A lumbar puncture

was performed, and the cerebro-spinal fluid was found to be under normal pressure, with a glucose content of 58 mg. per 100 ml., a protein content of 60 mg. per 100 ml. and a normal Lange colloidal gold curve. X-ray films of the cervical and thoracic segments of the spine showed evidence of osteoarthritis, particularly affecting the disc spaces between the fifth and sixth and between the sixth and seventh cervical vertebræ, and between the seventh and eighth and between the ninth and tenth thoracic vertebræ.

After his admission to hospital, the patient was maintained on a diet that simulated his usual home diet as closely as possible. He received no added vitamin B_{12} until the ninth day after his admission, when radioactive vitamin-B_{12} absorption tests were commenced, during which he received a total of 4000 μg of vitamin B_{12} by intramuscular injection. No further tenderness over the third to fifth thoracic vertebræ was noted after the third day in hospital. From his first day in hospital the patient felt better, but no objective neurological improvement was noted until two weeks after his admission (and one week after the commencement of vitamin B_{12} therapy), when his power was found to be improved and his spasticity to be diminished. At the time of his discharge, 25 days after admission, he was considerably better, but there was still some weakness and increased tone of the lower limbs.

At the time of his admission to hospital, the principal diagnoses considered were neoplastic involvement of the spinal cord, transverse myelitis and subacute combined degeneration of the cord. The subsequent improvement tended to exclude the first of these diagnoses, but it remains difficult to differentiate between the remaining two.

Comments

In both cases described here the patients had been vegans for several years, and had very low serum vitamin-B_{12} levels, with normal radioactive vitamin B_{12} absorption. They are therefore considered to have had nutritional vitamin-B_{12} deficiency.

It is held that vitamin-B_{12} deficiency can lead to detectable histological changes in the mucosal lining of the alimentary tract (Reisner, 1961; Boen *et alii*, 1958). Campbell and Dickinson (1960) note that the improvement of gastric acid secretion in some juvenile patients with pernicious anæmia treated with cyanocobalamin has led to the suggestion that the failure of gastric secretion may be, at least in part, the result rather than the cause of vitamin-B_{12} deficiency. The gastric biopsies in both cases presented here showed atrophic and inflammatory changes within the gastric mucosa. Similar gastric changes were described by Harrison, Booth and Mollin (1956) as occurring in a woman, aged 77 years, thought to be suffering from nutritional vitamin-B_{12} deficiency. Smith (1962), on the other hand, reported the case of a woman who had been a vegan for 18 years, and who for six years had been known to have a serum vitamin-B_{12} level of less than 60 $\mu\mu$g per ml., but the gastric biopsy findings were considered to be within normal limits.

It is interesting to note that the patient in Case I presented with anæmia, and had a serum folic-acid level within the normal range. On the other hand, the patient in Case II was not anæmic and had a high serum folic-acid level.

Summary

Two cases of nutritional vitamin-B_{12} deficiency occurring in Western Australia are described. It is believed that these are the first reported cases of this condition in Australia.

Acknowledgements

The two patients presented here were admitted to the hospital under the care of Dr. E. R. Beech, and we are grateful for his permission to publish their reports.

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Gastrointestinal Absorption, Plasma Transport, Surface Distribution, and Urinary and Fecal Excretion of Radioactive Vitamin B_{12} in Iron Deficiency

By J. D. Cook and L. S. Valberg

In 1933 Hartfall and Witts[1] reported that gastric juice obtained from some women with simple achlorhydric anemia and histamine-fast achlorhydria contained small amounts of intrinsic factor. With modern methods of investigation, Badenoch, Evans and Richards[2] found that the absorption of radioactive vitamin B_{12} was impaired in some iron-deficient subjects with gastric atrophy whereas in others with similar histologic involvement of the stomach it was within normal limits. Recently Cox and co-workers[3] have suggested that the defect in vitamin B_{12} absorption in certain patients with iron deficiency might be reversible in view of their findings that subnormal levels of serum B_{12} gradually returned to normal after iron therapy in a group of patients in whom the gastric secretory status was not completely determined. Studies in the rat indicate that iron deficiency produces a reversible defect in the secretion of intrinsic factor without severe reduction in the secretion of gastric acid or change in the histology of the gastric mucosa.[4]

The object of the present study was to determine firstly whether the secretion of intrinsic factor is impaired in iron-deficient subjects in whom gastric acid secretion is normal and secondly, to determine whether the low serum B_{12} levels described by Cox and co-workers might be due to deranged plasma transport of vitamin B_{12}, abnormal distribution of vitamin B_{12} in the body or to increased urinary or fecal excretion of the vitamin.

Materials and Methods

Selection of Subjects

Six control subjects were selected from cooperative psychiatric patients who were free from organic disease and who were found on investigation to have a hemoglobin concentration above 13 Gm./100 ml., normal peripheral blood smear, normal serum iron concentration[5] and normal gastric acid secretion as determined by the augmented histamine test.[6] Four iron-deficient subjects with normal gastric acid secretion were studied. The diagnosis of iron deficiency was based on hypochromia and microcytosis of the erythrocytes on peripheral blood smear, serum iron concentration less than 40 μg./100 ml., absence of stainable iron in the bone marrow and subsequent correction of the anemia by iron therapy. No histologic abnormalities were found in per-oral gastric biopsies in either the control or iron deficient group. The clinical and laboratory data on each subject are given in table 1.

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Table 1.—*Clinical and Laboratory Data in Control and Iron-Deficient Subjects*

Subject	Sex	Age	Etiology of Iron-Deficiency	Hb (Gm./100 ml.)	MCHC (%)	Serum Iron (μg./100 ml.)	Transferrin Saturation (%)	Augmented Histamine Test: pH	Augmented Histamine Test: Acid Production (mEq. HCL/Hour)
				Control					
C. S.	M	60	—	13.7	35.1	100	25.8	—	—
W. F.	M	75	—	14.4	35.1	78	24.1	1.8	5.1
S. R.	M	16	—	14.2	33.0	160	38.7	1.6	8.0
O. L.	F	38	—	15.0	34.1	93	28.5	1.2	15.5
H. B.	F	52	—	14.4	33.9	115	41.1	1.5	14.7
E. V.	F	34	—	14.1	34.4	97	45.8	1.1	14.5
Mean		46		14.3	34.3	107	34.0	1.4	11.6
				Iron-Deficient					
L. A.	F	26	Menorrhagia	5.0	25.0	—	—	1.9	2.8
G. M.	M	29	Bled from peptic ulcer	8.3	27.2	33	7.6	1.6	12.1
V. S.	F	34	Menorrhagia	10.4	30.1	30	9.5	1.8	20.0
L. D.	M	62	Bled from hiatus hernia	8.7	29.0	20	5.7	1.4	10.4
Mean		38		8.1	27.8	28	7.6	1.6	11.3

Serum Vitamin B_{12}

The vitamin B_{12} concentration of serum was measured by a microbiologic technic employing *Euglena gracilis*.[26]

Plasma Transport, Body Surface Distribution, and Urinary and Fecal Excretion of Radioactive Vitamin B_{12}

After an overnight fast, the subjects were given an intravenous injection of 5 μc. of Co^{58}–B_{12} (Philips Duphar, Holland) of high specific activity (10.2–77.1 μc./μg.). The dose of vitamin B_{12} varied from 0.07 to 0.48 μg. with mean values of 0.36 and 0.27 μg. for the control and iron-deficient groups, respectively. The radiovitamin, which in all cases was used within 1 month of production, had a radiochemical purity more than 95 per cent as determined by paper chromatographic technics.[7] Blood samples were obtained at 3 and 6 minutes, 3 hours and at daily intervals for 10 days to determine the disappearance of the labeled vitamin from the plasma. The blood was centrifuged and 4 ml. of plasma were counted in a small well-type sodium iodide crystal. Because of the low plasma activity in the last few days of the study counting errors as high as 10 per cent were encountered although the majority of specimens were counted with an error less than 5 per cent. The results were expressed as a percentage of the injected dose remaining in the plasma volume, the latter being calculated from the plasmatocrit and from the blood volume which was estimated by the formula of Allen and co-workers.[8] Surface measurements of radioactivity over the liver, spleen, sacral marrow and left kidney were made with a collimated sodium iodide crystal at 2- to 3-day intervals and the counting rate was expressed in relation to that of a standard prepared at the time that the dose was injected. Daily collections of urine and feces were carried out in the Special Investigation Unit of the Kingston General Hospital to ensure completeness of collection. Radioactivity in the specimens was assayed in a large plastic scintillation counter.[9] The counting error for urine samples was less than 4 per cent and for fecal specimens containing more than 0.1 per cent of the injected dose the error was less than 1.5 per cent. Both the fecal and urinary radioactivity were expressed as a percentage of the administered dose.

Table 2.—*Serum Vitamin B_{12} Levels, Co^{60}-B_{12} Absorption and Co^{58}-B_{12} Excretion in Controls and Iron-Deficient Subjects*

Subject	Serum B_{12} ($\mu\mu$g./ml.)	Co^{60}-B_{12} Absorption (%)	Co^{58}-B_{12} Excretion (% Injected Dose)		
			Fecal Day 0–9	Urinary Day 0	Urinary Day 1–9
		Controls			
C. S.	364	29.3	2.55	2.94	1.65
W. F.	140	25.4	2.41	3.08	1.49
S. R.	264	21.0	1.59	3.44	1.96
O. L.	320	7.8	1.48	8.65*	1.45
H. B.	155	17.4	1.56	2.35	0.99
E. V.	212	26.4	1.11	2.04	0.93
Mean	243	21.2	1.78	2.77	1.41
		Iron-Deficient			
L. A.	475	29.8	1.24	5.46	2.10
G. M.	440	23.8	2.61	6.49	1.30
V. S.	289	35.0	2.00	5.98	2.30
L. D.	560	28.5	3.35	6.70	1.82
Mean	441	29.3	2.30	6.16	1.88

*See text—not included in calculation of mean.

Gastrointestinal Absorption of Co^{60}-Vitamin B_{12}

The Schilling technic[10] was employed to determine the absorption of a 0.5 μg. dose of Co^{60}-B_{12} (Abbott Laboratories, Oakridge, Tenn.) on the tenth day of the study. Four ml. of urine were counted in a sodium iodide well equipped with a single channel pulse-height analyzer. From the predetermined pulse height distribution spectrum of each cobalt isotope, the contribution of residual Co^{58} radioactivity to the Co^{60} photopeak counting rate was calculated by the method of Reizenstein et al.[11] The reproducibility of the method was evaluated by determining the Co^{60} content of 10 samples containing a known amount of Co^{60} and varying levels of Co^{58} similar to those encountered in the present study. The mean Co^{60} activity prior to the addition of Co^{58} was 250.8 cpm. and the mean activity determined by differential counting was 251.7 cpm. The standard deviation of the results calculated from the difference between each sample was 3.97 cpm.

Results

Absorption of Co^{60}-B_{12}

The percentage of the oral dose of Co^{60}-B_{12} excreted in the urine during the first 24 hours was within normal limits in all of the subjects, with a mean of 21 per cent in the controls and 29 per cent in the iron-deficient subjects (table 2).

Serum Vitamin B_{12}

The serum levels were within normal limits in both control and iron-deficient subjects (table 2).

Plasma Disappearance of Co^{58}-B_{12}

There was a significant delay in the disappearance of radioactivity from the plasma of the iron-deficient subjects. The mean percentage of the injected

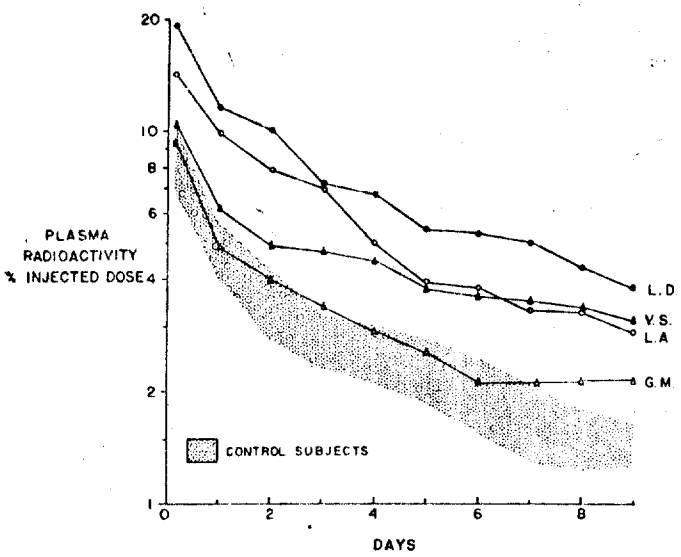

Fig. 1.—Plasma disappearance of radioactivity expressed as a per cent of the injected dose in total plasma volume after an intravenous injection of Co^{58}-B_{12} in controls and iron-deficient subjects.

dose remaining in the total plasma volume at 3 minutes in the iron-deficient subjects was 52 per cent compared to a mean of 36 per cent in the controls, and at 6 minutes the mean values were 36 per cent and 27 per cent respectively. The difference between the two groups at both times was statistically significant ($p < 0.01$). The plasma radioactivity remaining at 3 hours and at daily intervals for 10 days is shown in figure 1. The differences between the controls and iron-deficient subjects were significant at the 5 per cent level on days 3, 5, 7 and 9. If the results in each group from day 1 to day 9 are considered as a single sample the difference between the two groups is highly significant ($p < 0.001$). The residual plasma radioactivity was not correlated with the dose of Co^{58}-B_{12}, the serum B_{12} concentration or the urinary and fecal excretion of the tracer in either the control or iron-deficient subjects.

When the disappearance of radioactivity was expressed as the amount remaining in 1 ml. of plasma no significant difference was observed between the control and iron-deficient subjects 3 minutes and 6 minutes after the injection of Co^{58}-B_{12}. The mean percentage of the dose remaining in 1 ml. of plasma at 3 minutes in the iron-deficient subjects was 176×10^{-4} per cent compared to a mean of 173×10^{-4} per cent in the controls and at 6 minutes the mean values were 120×10^{-4} per cent and 125×10^{-4} per cent, respectively. The mean percentage radioactivity remaining in 1 ml. of plasma at 3 hours and at daily intervals for 10 days in the controls and iron-deficient subjects is shown in figure 2. There was a delay in the plasma clearance of radioactivity in 3 out of the 4 iron-deficient subjects. Statistical analysis of the results on each day showed no significant difference between the controls and the iron-deficient groups but if the results in each group from day 1 to

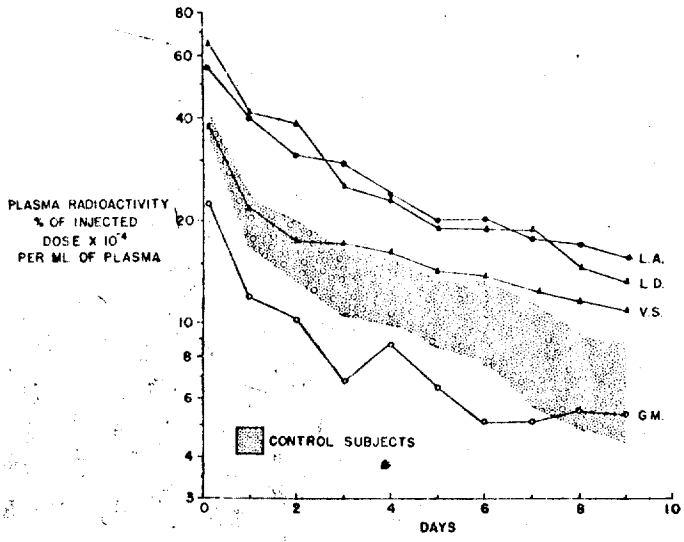

Fig. 2.—Plasma disappearance of radioactivity expressed as a per cent of the injected dose remaining in 1 ml. of plasma in controls and in iron-deficient subjects following an intravenous injection of Co^{58}-B_{12}.

day 9 are considered as a single sample the difference between the two groups is significant ($p < 0.01$).

Organ Distribution of Co^{58}-B_{12}

Surface measurements of radioactivity in 4 control subjects and 3 patients with iron-deficiency showed no appreciable difference between the two groups (fig. 3). In each group the radioactivity over the sacrum, spleen and left kidney remained relatively constant while the hepatic radioactivity showed a gradual increase of approximately 40 per cent between day 1 and day 9.

The Excretion of Co^{58}-B_{12}

The *pattern* of urinary and fecal excretion of radioactivity was similar in both the control and iron-deficient groups. From day 0 to day 2 excretion occurred mainly in the urine, from day 3 to day 5 fecal excretion was greater, and from day 6 to day 9 it was about equally distributed between urine and feces. The percentage of the injected dose excreted in the urine in the first 24 hours was roughly twice the total amount excreted during the last 9 days of the study. The mean excretion in the first 24 hours in control subjects was 2.77 per cent compared to 6.16 per cent in the iron-deficient group. The difference was statistically significant ($p < 0.01$). In calculating the mean value of the control group, (O. L.) who excreted more than twice the radioactivity observed in the other subjects was rejected from the group using the criteria of Grubbs.[12] There was no difference between the control and iron-deficient groups in either the total fecal excretion ($p > 0.20$) or total urinary excretion ($p > 0.10$) from day 1 to day 9 inclusive (table 2).

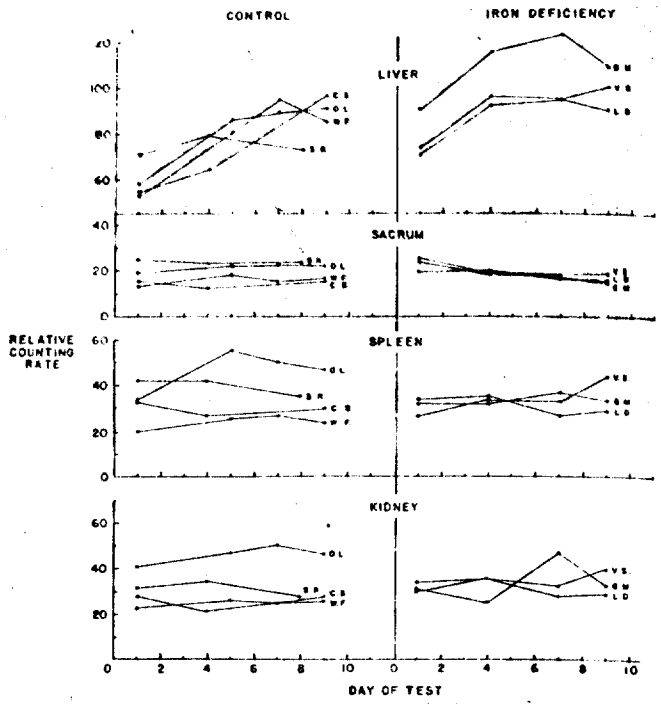

Fig. 3.—Surface radioactivity over liver, sacrum, spleen and kidney in control and iron-deficient subjects following intravenous injection of Co^{58}-B_{12}.

Discussion

Since Co^{58}-B_{12} clearance from the plasma follows a series of exponential curves one would expect the rate of clearance per unit volume to be equal if the processes were similar. The delay in plasma clearance of the labeled vitamin seen in our iron-deficient patients 3 minutes and 6 minutes after injection of Co^{58}-B_{12} can be accounted for by the increased plasma volume rather than by iron deficiency per se because the results expressed per ml. of plasma are similar in the two groups. It is clear that the slight delay in clearance observed in one of the iron-deficient subjects (G. M.) between day 1 and day 9 (fig. 1) is due to the increased plasma volume but this does not appear to account for the delayed clearance in the other subjects (fig. 2). It is of interest that symptoms of anemia were of shorter duration in G. M. than in the other subjects. Delayed plasma clearance of vitamin B_{12} has been observed in chronic myelogenous leukemia[13,14] and in pernicious anemia[14,15] but there was no evidence of either of these disorders in our patients. In theory there are three possible explanations for delayed clearance. Firstly, it may be due to an increased rate of recycling of vitamin B_{12} between tissues and plasma. Secondly, an abnormality of the binding of vitamin B_{12} to plasma protein might be responsible. This could be due to an increase in the protein fraction to which vitamin B_{12} is normally bound or to binding of the vitamin to other plasma proteins from which it is cleared at a slower rate than normal. For

instance it might be bound to transferrin, which was increased in the plasma of the iron-deficient subjects. Thirdly, diminished uptake of vitamin B_{12} by the tissues, secondary to decreased erythropoiesis might be responsible for delayed clearance. It is not possible to decide on the basis of the current evidence which of these mechanisms, if any, is responsible for delayed clearance in iron deficiency. It should be pointed out that a uniform dose of 5 μc. of Co^{58}-B_{12} was given to each patient to ensure that the radioactivity would be sufficient to provide accurate measurement. The dose of vitamin B_{12} which was administered to the subjects varied from 0.07 μg. to 0.48 μg. The absence of correlation between the amount of vitamin B_{12} injected and the rate of disappearance of radioactivity from the plasma is in agreement with results of Ritz and Meyer[13] and Hall et al.[16] who also found that plasma disappearance was unaffected by the amount of vitamin B_{12} injected when the dose was less than 1 μg.

Although the Co^{58}-B_{12} which was administered was of the highest specific activity available the average amount of vitamin B_{12} given to the control and iron-deficient subjects was equivalent to roughly 30 per cent of the total plasma vitamin B_{12}. The high level of radioactivity found in the urine in both the control and iron deficient subjects in the first 24 hours represents an unphysiologic excretion of excess vitamin. The higher urinary excretion in iron-deficient subjects during this period suggests less complete or a lower rate of B_{12} binding by iron-deficient plasma in view of the fact that vitamin B_{12} bound to plasma protein is not excreted by the kidney whereas free or unbound vitamin B_{12} is rapidly filtered by the glomerulus.[17] The difference in urinary excretion between control and iron-deficient subjects is too large to be explained by an altered glomerular filtration rate which if anything is reduced in anemic states. The absence of a significant difference between normal and iron-deficient subjects in either the urinary or fecal excretion from day 1 to day 9 suggests that excessive excretion of vitamin B_{12} does not occur when physiologic amounts of vitamin B_{12} are present. However there is a possibility that complete labeling of body vitamin B_{12} did not occur in the present study and an extended period of observation might be required to exclude minor differences in excretion.[18]

The observation by Badenoch and co-workers[2] of impaired vitamin B_{12} absorption in iron-deficient subjects with gastric atrophy, together with our findings of normal vitamin B_{12} absorption in iron-deficient subjects with normal gastric secretion, suggests that in man the occurrence of defective secretion of intrinsic factor in iron deficiency is due to gastric atrophy rather than to a biochemical defect secondary to iron deficiency. This is in contrast to findings in the rat where iron deficiency produces a defect in intrinsic factor secretion unaccompanied by severe abnormality in the gastric secretion of acid or pepsin or alteration in the histology of the gastric mucosa.[4] Whether the chronic gastritis commonly found in patients with iron deficiency is the result or the cause of the iron deficiency in these patients remains to be settled.

The report of Cox and associates[3] of low serum vitamin B_{12} levels in 13

of 25 iron-deficient subjects and the gradual return of these values to normal after iron therapy has been cited as evidence that iron deficiency produces a defect in intrinsic factor secretion which is corrected by iron. Their observations deserve critical analysis. Firstly, 11 of their 13 subjects had in addition to iron deficiency, partial gastrectomy, steatorrhea, inadequate diet, and regional ileitis, disorders which in themselves could lead to a low serum vitamin B_{12}. Secondly, their assumption that low serum B_{12} levels always reflect diminished absorption is not justified. Recently Jones and associates[19] have reported defective vitamin B_{12} absorption in only 50 per cent of postgastrectomy subjects with low serum B_{12} levels. Thirdly, their assumption that the gradual increase in serum B_{12} after iron therapy is due solely to correction of impaired vitamin B_{12} absorption is unwarranted. For example, low serum B_{12} levels in steatorrhea may rise to normal after 3 or 4 days of folic acid therapy alone although the absorption of vitamin B_{12} remains defective.[20] The conclusion of Cox and co-workers that iron deficiency produces a defect in intrinsic factor secretion which is corrected by iron is not supported by their evidence or by the results of this study. However, the patients in the present study differed from those investigated by Cox and co-workers in that the serum B_{12} levels were normal, and further investigation will be required to determine whether iron-deficient patients either with low serum B_{12} levels or vitamin B_{12} levels which rise after iron therapy have defective intrinsic factor secretion. It might be preferable to study the absorption of vitamin B_{12} from food in these subjects rather than absorption of the vitamin in the fasting state.

Hydrochloric acid has been found to reappear in the gastric juice of some young men[22,23] and infants[24] after the correction of anemia by iron,[1,21] but peroral biopsies from iron-deficient patients with histologic abnormalities in the gastric mucosa have not shown improvement in the appearance of the gastric lesion after iron therapy.[21,25] It is probable that defective intrinsic factor secretion associated with gastric atrophy is irreparable but follow-up studies of vitamin B_{12} absorption in treated patients with iron deficiency who have previously had impaired intrinsic factor secretion are needed to settle the question.

Summary

No evidence of vitamin B_{12} deficiency or of any mechanism which might lead to vitamin B_{12} deficiency, such as defective absorption or increased urinary or fecal excretion of the vitamin, has been found in iron-deficient subjects in whom gastric acid secretion and gastric biopsies were normal. It is concluded that when vitamin B_{12} deficiency occurs in iron-deficient subjects it is the result of gastric atrophy. An unexplained finding was delayed disappearance of an intravenous dose of Co^{58}-B_{12} from the plasma in iron-deficient subjects.

Summario in Interlingua

Nulle evidentia de carentia de vitamina B_{12} e nulle mechanismo que poterea resultar in un tal carentia—i.e., e.g., un defective absorption o un augmentate excretion urinari o fecal del vitamina—esseva trovate in subjectos a carentia

de ferro in qui le secretion gastric de acido e biopsias gastric esseva normal. Es concludite que le carentia de vitamina B_{12} que occurre non infrequentemente in subjectos a carentia de ferro es le resultato de un atrophia gastric. Un inexplicate constatation esseva le retardate disparition ab le plasma in subjectos a carentia de ferro de un dose intravenose de vitamina B_{12} marcate con Co^{58}.

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The vitamin B_{12}, folic acid and ascorbic acid content of human bone marrow cells.

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For a better understanding of disorders of erythropoiesis, it is desirable to know the bone marrow content of certain haemopoietic factors. The work reported here was undertaken to establish normal values for vitamin B_{12} activity, folic acid activity and ascorbic acid in the marrow cells.

Table 1. The mean cell content of vitamin B_{12} activity, folic acid activity and ascorbic acid in twelve control subjects

(The range is given in parentheses.)

Concentration per unit volume of packed cells			Content per million cells		
B_{12} activity (pg/ml.)	FFA (mμg/ml.)	Ascorbic acid (mg/100 ml.)	B_{12} activity (pg)	FFA (mμg)	Ascorbic acid (μg)
12,000	347	13·1	25·8	0·60	0·24
S.E. 1,265	S.E. 57	S.E. 0·83	S.E. 3·1	S.E. 0·10	S.E. 0·04
(6,900–19,800)	(95–726)	(6·5–16·6)	(9·9–44·0)	(0·2–1·2)	(0·11–0·46)

Samples of 1–2 ml. of marrow were taken by needle puncture of the sternum from twelve volunteers—ten medical staff and students and two hospital patients in good general condition and without haematological abnormalities. The samples were heparinized, and centrifuged in Wintrobe tubes at 3000 rev/min for 30 min. After noting the volume of the layer of marrow cells, this was removed and the cells evenly suspended in 6–10 ml. of 0·85% saline. A nucleated cell count was made on the suspension, and the remainder was used for estimation of vitamin B_{12} activity with *Lactobacillus leichmanii* (Meynell, Cooke, Cox & Gaddie, 1957), folic acid activity (FAA) with *Streptococcus faecalis* by a modification of the method of Teply & Elvehjem (1945), and ascorbic acid by the method of Roe & Keuther (1943). Siliconed glassware was used in collecting the samples and making the cell suspensions.

c

The results are shown in Table 1; they are expressed as concentration per unit volume of packed marrow cells, and as content per million cells. The work of Wolff, Royer & Karlin (1951) suggests that 85–95% of the B_{12} activity represents true vitamin B_{12}. Comparison of the results with published data shows that the concentrations of B_{12} activity and FAA are several times higher in marrow cells than in peripheral white cells, though that of ascorbic acid is lower. The concentrations of B_{12} activity and FAA are only 2–5% and about 10% respectively, of those in liver. Consequently, it appears that the contribution of the bone marrow to the total vitamin B_{12} and folic acid and related substances in the body is relatively small.

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Complex of intrinsic factor and B_{12} in human ileum during vitamin B_{12} absorption[1]

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COOPER, BERNARD A. *Complex of intrinsic factor and B_{12} in human ileum during vitamin B_{12} absorption.* Am. J. Physiol. 214(4): 832-835. 1968.—During absorption studies in man, the size and biological characteristics of at least some of the ^{57}Co-labeled vitamin B_{12} found in extracts of ileum were identical with those of a complex of human intrinsic factor (IF) and vitamin B_{12}. Although the $^{57}CoB_{12}$ in the ileum combined with antibody to IF, its behavior during combination differed from that of normal human gastric juice with vitamin B_{12}. The IF-B_{12} complex (mol wt approximately 60,000) becomes intimately associated with the wall of the ileum during absorption of vitamin B_{12} in vivo and may enter the wall itself. During this process its biological properties are unchanged, but its immunological characteristics appear to be modified.

transport across intestine; gastric intrintic factor

ABSORPTION of low concentrations of vitamin B_{12} from the gastrointestinal tract requires combination with gastric intrinsic factor (IF). Absorption is slow (2, 5, 7), and studies with animals indicate that during this process an intact complex of IF-vitamin B_{12} becomes intimately associated with the wall of the small intestine and may actually enter it (3, 15, 16). Since the intrinsic factor molecule is antigenic, and large, incorporation of an intact complex IF-vitamin B_{12} into the intestinal wall during absorption would represent an unusual form of transport, and one which would expose the organism to antigenic material.

To determine whether an intact complex of these two substances becomes intimately associated with the wall of the ileum during vitamin B_{12} absorption in man, we studied the characteristics of the vitamin associated with the wall of the ileum during absorption of radioactive vitamin B_{12}; its physical, biological, and immunological characteristics were compared with those of vitamin B_{12} bound to human gastric IF. A preliminary report of these findings has been published (4).

MATERIALS AND METHODS

Vitamin B_{12}-^{57}Co (Charles E. Frosst, Montreal) was verified as cyanocobalamin by its partition coefficient between benzyl and aqueous buffer (11). Its specific activity was 15–30 $\mu c/\mu g$.

The subjects studies were two elderly women who had carcinoma of the colon which necessitated removal of segments of normal ileum also. They ingested 0.5 μg of ^{57}Co-labeled vitamin B_{12}; subsequently, both were shown competent to absorb this. The resected tissue, which was obtained 10 and 12 hr after the feeding, comprised a segment of terminal ileum attached to ascending colon. The segment, which was reported to appear normal when examined histologically, was detached and rinsed twice in 500 ml of ice-cold isotonic saline containing 0.01 M sodium phosphate at pH 7.4. Two aqueous extracts were prepared from each ileum: one was made from material obtained by scraping the surface of the washed ileum with a glass slide (scrapings extract), and the other from the remainder (residue extract). Mucosal tissue probably was present in both, in higher percentage in the scrapings extract, since no attempt was made to remove all of the mucosa by scraping.

The scrapings and residue were homogenized separately with 50 ml of isotonic saline in a VirTis 45 homogenizer and centrifuged for 30 min at 9,000 × g. The amber, slightly opalescent supernatant solutions contained 70–80% of the total radioactivity in the washed segment of ileum, which in turn represented 3.8–5.2% of the ingested radioactive B_{12}. The $^{57}CoB_{12}$ content of the scrapings extracts from the two subjects was 0.68 and 0.150 ng/ml, and the residue extracts contained 0.10 and 0.15 ng/ml. Assay with *Euglena gracilis* showed a total B_{12} content of 0.50 and 8.0, and 2.0 and 15.0 ng/ml, in the two preparations in the two subjects.

Filtration through Sephadex utilized a glass column 100 cm in height and 2.5 cm in diameters which was perfused from below upwards. The column was maintained at 4–6 C with a water jacket and samples were

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collected with a drop counter. The column was standardized with bovine albumin, albumin dimer, alpha-lactalbumin, and deoxyribonuclease filtered in isotonic saline containing 0.01 M sodium phosphate pH 7.4. For the immunological studies, a small column of Sephadex G-150 in a 10-ml pipet was used (4).

Vitamin B_{12} absorption in vivo was determined with the Schilling test utilizing two intramuscular injections of 1 mg of cyanocobalamin at 1.5 and 25.5 hr after feeding the material containing radioactive vitamin B_{12}. Urine was collected for 48 hr. The tests were carried out in a single volunteer subject who had undergone total gastrectomy some years before, and whose absorption and excretion of graded doses of vitamin B_{12} and human gastric juice had been determined previously and were reproducible (17).

Determination of intrinsic factor activity in vitro utilized the guinea pig intestinal mucosal homogenate assay of Sullivan, Herbert, and Castle (13).

Reaction between intrinsic factor-B_{12} and antibody to this complex was determined using precipitation of serum globulin with ethanol as described previously (6).

OBSERVATIONS

The extracts were filtered through Sephadex G-150 to determine the approximate number and size of B_{12}-binding materials present. They were centrifuged at 30,000 × *g* for 3 min, and the optically clear supernatant fluids were filtered. Distribution of radioactivity, optical density and vitamin B_{12} content in the fractions were determined (Fig. 1). The homogenized scrapings were used for this experiment, but the pattern was essentially identical with that obtained during filtration of residue extracts. Radioactivity in the effluent appeared in two distinct fractions: one filtered with an apparent molecular weight of 90,000–110,000 and the second of 50,000–60,000, compared with standards of bovine albumin, albumin dimer, alpha-lactalbumin, and deoxyribonuclease. The major B_{12}-binding fraction in samples of gastric juice from normal human subjects and from one

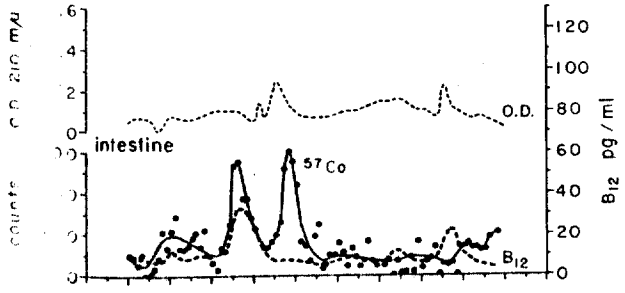

FIG. 1. Filtration of an extract of scrapings of human ileum through Sephadex G-150. Tube number, from *1* to *120*, is indicated on the horizontal axis. Each tube contained 5.87 ml of effluent. Solid line indicates the pattern of radioactivity (counts/10 min) in the effluent, and the dark spots the radioactivity in each tube. Broken lines indicate the optical density of the effluent and its total B_{12} concentration assayed microbiologically. With the $^{57}CoB_{12}$ administered, 200 counts represent 1 pg of $^{57}CoB_{12}$.

TABLE 1. *Uptake of vitamin B_{12} from intestinal extract by homogenate of guinea pig intestinal mucosa*

Source of Vitamin B_{12}	Mean Uptake by Homogenate (n = 4), pg/flask
^{57}Co in gut	3.81±1.25
^{57}Co in gut, boiled	0.91±0.64
$^{58}CoB_{12}$ + normal human gastric juice	3.45±1.3
$^{58}CoB_{12}$	0.41±0.35

of patients filtered with a mobility identical with that of the second fraction shown in Fig. 1.

To determine whether the B_{12}-binding material in human ileum would function in vivo as gastric IF, a patient who had undergone total gastrectomy some years before was given 20 ml of residue extract which contained 0.003 μg of $^{57}CoB_{12}$ and 0.3 μg of total B_{12} activity. Eighty percent of the radioactivity in this extract filtered through Sephadex with an apparent molecular weight of 50,000–60,000. The extract was fed twice; the second time, 72 hr later, 0.003 μg of $^{58}CoB_{12}$ was added. The radioactivity flushed into the urine in 48 hr by two injections of 1 mg of nonradioactive B_{12} was determined. The urine contained 57.12% and 67.20% of the $^{57}CoB_{12}$ administered, but none (< 0.00%) of the added free $^{58}CoB_{12}$. When 0.3 μg of $^{57}CoB_{12}$ was fed, with normal human gastric juice as a source of IF, 53.44% of administered radioactivity was excreted in the urine. During previous studies of this patient, the amount of radioactive B_{12} which appeared in the urine was 0.21% of a dose of 0.5 μg, 10.0% when 1 ml of normal gastric juice was added and 19–26% when 2–20 ml of gastric juice was added to the $^{57}CoB_{12}$. Thus, the $^{57}CoB_{12}$ in the residue extract was absorbed as if it were a mixture of $^{57}CoB_{12}$ and IF, whereas free $^{58}CoB_{12}$ mixed with this extract was not absorbed.

The extracts of intestine were tested to determine whether the radioactive material in them functioned as IF in vitro as well as in vivo, by measuring the uptake of radioactivity from the extract by insoluble material in homogenized guinea pig ileum (11). In this assay a larger percentage of B_{12} bound to IF than of free B_{12} or B_{12} bound to materials without IF activity adsorbs to insoluble material in the homogenate of guinea pig intestine. The uptake of $^{57}CoB_{12}$ activity from the extract was compared with that of identical concentrations of free $^{58}CoB_{12}$ and of $^{58}CoB_{12}$ bound to the IF of normal human gastric juice. Extract was put into all of the incubation flasks, and $^{58}CoB_{12}$, alone, or preincubated with gastric juice, was added to some. The uptake of each isotope by the guinea pig homogenate was determined (Table 1). The homogenate absorbed similar amounts of $^{57}CoB_{12}$ from the extract and from $^{58}CoB_{12}$ with gastric juice, but less free $^{58}CoB_{12}$, with and without extract. After the extract had been boiled, its $^{57}CoB_{12}$ activity was no longer taken up by the homogenate. This finding differentiates the activity from thermostable blood-group substance, the only material other than IF

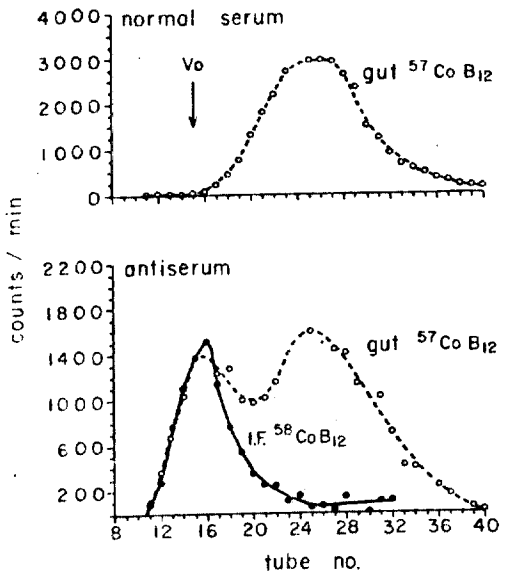

FIG. 2. Effect of antiserum on filtration through Sephadex G-150 of $^{57}CoB_{12}$ from an extract of ileal scrapings and of $^{58}CoB_{12}$ bound to human intrinsic factor. Upper figure indicates the pattern of radioactivity in the effluent solution when the extract was filtered mixed with 0.1 ml of normal serum. In the lower figure, $^{57}CoB_{12}$ activity is indicated by the broken line, and that of $^{58}CoB_{12}$ bound to human intrinsic factor by the solid line. In this experiment, before filtration, the mixture of extract and $^{58}CoB_{12}$ bound to intrinsic factor was mixed with 0.1 ml of serum containing antibody to human intrinsic factor. Each tube contained 5 drops of effluent; the total volume of the column was 10.5 ml.

that has been reported to enhance B_{12} uptake in this test (9). Thus, the $^{57}CoB_{12}$ activity in the intestine had the biological characteristics of vitamin B_{12} bound to IF in vivo and in vitro.

Intrinsic factor can be identified immunologically also by its reaction with the antibodies that are present in the serum of about 50% of patients with pernicious anemia (1, 10). If the ^{57}Co activity in the intestinal extract was bound to IF, it would be expected to combine with such antibodies to intrinsic factor. This reaction could be detected by observing increase in molecular size of the ^{57}Co material during Sephadex filtration.

The scrapings extracts were mixed with normal serum and with antiserum, and the resulting solutions were filtered through a small column of Sephadex G-150 which provided rapid filtration and reduced dissociation of the antigen-antibody complex (6). As a control, a small quantity of $^{58}CoB_{12}$ (17 $\mu c/\mu g$) bound to normal human gastric juice was added to the extract before mixture with the antiserum. The complex of IF-$^{58}CoB_{12}$ was prepared by adding an excess of $^{58}CoB_{12}$ to gastric juice; unbound B_{12} was removed with charcoal (8). The effect of incubation with serum on the pattern of radioactivity in the effluent fractions from the column is shown in Fig. 2. The two fractions which were observed during experiments with the larger column could not be differentiated in the effluent from the small column. When antiserum was mixed with the extract, about 30% of the ^{57}Co radioactivity and all of the ^{58}Co activity were excluded from the gel, indicating increase in molecular weight, apparently due to combination of the radioactive material with antibody. Thus, at least 30% of the ^{57}Co activity was bound to material with the immunological characteristics of IF. During filtration of this extract through the large column of Sephadex G-150, 7[illegible]% of the radioactivity was found in the fraction with apparent molecular weight of 50,000–60,000.

When antibodies combine with IF, the combination can be precipitated from solution with ethanol, ammonium sulfate (10), of antihuman-globulin serum (14). When $^{57}CoB_{12}$ bound to gastric juice was incubated with antiserum, and the globulin fraction precipitated with 45% ethanol (4), 35.08 ± 20.8% of the radioactivity appeared in the washed precipitate, whereas only 5.6 ± 5.4% appeared when incubation was with normal serum; 10.0 ± 6.6% and 10.0 ± 8.0% of free radioactive B_{12} was precipitated with ethanol when incubated with antiserum and normal serum, respectively. When the scrapings extract was mixed with antiserum, 27.0 ± 2.5% of its radioactivity was precipitated with ethanol, whereas 26.2 ± 1.5% was precipitated when incubation was with normal serum. Thus, in this procedure, no more ^{57}Co activity appeared in the precipitate after incubation with antiserum than with normal serum, indicating that, in this assay, the ^{57}Co material in the extract did not have the immunological characteristics of IF. This was not due to alteration of the molecule in vitro. Incubation of $^{58}CoB_{12}$ and human gastric juice with the extract for 18 hr at 4 C, and for 2 hr at 37 C, did not alter the immunological reactivity in these tests. Although 30% of the ^{57}Co activity in this extract was capable of combining with antibody to human IF (Fig. 2), this complex with antibody must have dissociated during precipitation with ethanol. The complex of intrinsic factor and vitamin B_{12} does not dissociate under these conditions (6). A similar phenomenon has been observed when antibody to human IF reacted with rat IF (6).

DISCUSSION

These data indicate that most of the radioactive B_{12} associated with ileum during B_{12} absorption in man is bound to material with the biologic properties of intrinsic factor. Human intrinsic factor and vitamin B_{12} can react with human ileum (12). Although no definite differentiation can be made between an IF-B_{12} complex intimately associated with the outside of the cells of the intestinal mucosa and one inside these cells, several facts favor the latter. These include the observation that the majority of the ^{57}Co activity not removed by exhaustive washing of the intact mucosa was found subsequently in the saline extract rather than associated with cell debris removed by centrifugation, and the observed disparity between biological and immunological characteristics of the $^{57}CoB_{12}$ in the intestine. This activity functioned biologically in vivo (Schilling test) and in vitro (homogenate

assay) as if a large proportion of it was bound to IF. Only a small proportion could be shown to combine with antibodies to human IF, however, and even that amount dissociated during precipitation of the complex with ethanol. This suggests that a portion of the IF molecule responsible for its immunological properties had been altered in some way. Similar alteration of the IF of human gastric juice was not observed during incubation in vitro, which may indicate some effect of transport or intestinal surface on the IF molecule in vivo.

One cannot completely exclude the possibility that all or a portion of the intrinsic factor-like activity recovered from the ileum was attached to microvilli during washing, and was freed during homogenizing. Such material, either freed from the microvilli or attached to broken microvilli would not have sedimented during centrifugation at 30,000 × *g* and so would have appeared in the supernatant solution. The apparent molecular size of both major fractions of radioactive material should exclude the possibility that the intrinsic factor-B_{12} molecule was associated with a significant fragment of microvillus, since this would have increased the molecular size to greater than 100,000, the apparent size of the fraction with the smallest elution volume through Sephadex. It is possible that the tiny fraction of radioactive material which filtered through sephadex with materials excluded from the gel (the first small hump in Fig. 1) could be explained by such a phenomenon. Whether the other fractions of radioactive material represent intrinsic factor-B_{12} which eluted from microvilli during preparation, and that no significant quantity of radioactive B_{12} was inside of the cells, cannot be determined.

The significance of the material with apparent molecular weight of 90,000 100,000 in the extracts of ileal scrapings is not clear. Whether it represents a polymer of the serum-binding protein "TC-II" cannot be evaluated at this time.

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Coexistence of Pernicious Anaemia and Chronic Myeloid Leukaemia: An Experiment of Nature Involving Vitamin B_{12} Metabolism

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SUMMARY. Chronic myeloid leukaemia (CML) and pernicious anaemia (PA) coexisted in a 51-yr-old man (who also had myasthenia gravis). His serum vitamin B_{12} level was normal instead of the high value expected in CML, or the low value expected in PA. Short-term culture of his peripheral blood cells showed deranged DNA synthesis of the type observed in vitamin B_{12} deficiency, indicating that his 'normal' serum vitamin B_{12} level was not associated with normal amounts of vitamin B_{12} available to his CML cells. A tracer dose of 0.45 μg of [^{57}Co]vitamin B_{12} disappeared abnormally slowly from his serum over 24 hr (as previously observed by others in patients with either CML or PA). A therapeutic injection of vitamin B_{12} appeared to disappear abnormally slowly from his serum over a period of 2 yr. His serum showed a markedly elevated unsaturated vitamin B_{12} binding capacity, with a less sharp increase in percentage of vitamin B_{12} binding α as compared to β globulin than usually expected in CML.

Withholding therapeutic vitamin B_{12} for 2 yr has been associated with stabilization of his WBC at the 40 000–50 000/μl range. Although this association could be chance rather than cause and effect, the patient does represent an 'experiment of nature' involving vitamin B_{12} metabolism and does raise the question as to whether his leukaemia is retarded by his deficiency of vitamin B_{12}, which results from inadequate absorption of vitamin B_{12} and possibly from elevated serum vitamin B_{12} binding α globulin rendering his circulating vitamin B_{12} metabolically inert.

The coexistence of pernicious anaemia (PA) and chronic myeloid leukaemia (CML) is a rare occurrence (Woolley, 1944; Sterne *et al*, 1941; Tawast & Siurala, 1956; Blackburn, 1957; Hitzenberger, 1958; Mosbech, 1959; Britt & Rose, 1966; Nielson & Jensen, 1970). This case report presents our observations over an 8 yr period on the metabolism of vitamin B_{12} in a patient with PA and CML.

CASE REPORT

First Admission (26–31 March 1962)

C.S., a 51-yr-old Negro man, was first admitted to The Mount Sinai Hospital in March 1962, complaining of weakness in the legs and blurred and occasional double vision. Physical examination revealed slight ptosis of the right eyelid. Complete blood count at the time revealed a haemoglobin of 14.4 g/100 ml, white blood count of 3800/μl, and a differential count of 63% segmented polymorphonuclear cells (PMN), 2% bands, 24% lymphocytes, 5% eosinophils and 6% monocytes. Blood chemistries, lumbar puncture, electrocardiogram, and LE preparation were unremarkable. Muscle biopsy revealed some fibre atrophy and was considered compatible with a diagnosis of myasthenia gravis. The patient's weakness responded dramatically to 'Tensilon'. He was treated with 'Mestinon' and discharged on it, to be followed in the Myasthenia Gravis Clinic.

Second Admission (23 May–16 July 1964)

The patient's second admission to the hospital was prompted by the finding of anaemia, slight leucocytosis and an abnormal differential count. On admission, he had a haemoglobin of 7.2 g/100 ml, with a PCV of 24% and a red cell count of $2.6 \times 10^6/\mu$l. Red cell indices were: mean corpuscular volume 92.5 fl, mean corpuscular haemoglobin 28.5 pg, and mean corpuscular haemoglobin concentration 31.5%. White cell count was 10 222 μl with a differential count of 67% segmented PMN, 5% bands, 2% myelocytes, 3% promyelocytes, 1% bands, 15% lymphocytes, 1% eosinophils and 6% monocytes. Three nucleated red cells were seen per 100 WBC. Platelet count was 122 000/μl; serum vitamin B_{12} 163 pg/ml; serum folate 6.1 ng/ml; serum iron 98 μg/100 ml; unsaturated iron binding capacity 262 μg/100 ml; neutrophil alkaline phosphatase score 14 (normal 25–100). Sickle cell preparation was negative; haemoglobin electrophoresis revealed haemoglobin A. A gastric analysis revealed no free acid and a Glass test (Glass, 1954) performed without intrinsic factor revealed no hepatic uptake. When the radioactive vitamin B_{12} was administered with intrinsic factor, the hepatic uptake became normal.

The patient was treated with 100 μg of vitamin B_{12} daily parenterally. He responded clinically and haematologically (reticulocytes up to 9.6%, increase in haemoglobin after a week on therapy) to this dose of vitamin B_{12} and was discharged on 16 July 1964 to be followed at the Hematology and Myasthenia Gravis Clinics. The patient was continued on 100 μg vitamin B_{12} monthly for several months, after which he was lost to follow-up.

When seen at the Emergency Room on 19 April 1968, he was found to have a haemoglobin of 5.5 g/100 ml, a lactate dehydrogenase of 1050 i.u./100 ml, and he was given an injection of 1000 μg of vitamin B_{12}. A serum vitamin B_{12} level drawn prior to the vitamin B_{12} injection was 151 pg/ml (see Table 1). Follow-up a week later revealed hepatosplenomegaly; an injection of 200 μg of vitamin B_{12} was given. The patient's haemoglobin increased to 13 g/100 ml, and he had a peak reticulocyte count of 12.5%. On 3 May 1968, WBC = 20 000/μl; on 7 June 1968, WBC = 35 000/μl; on 5 July 1968, WBC = 42 000/μl.

Third Admission (9–19 July 1968)

The patient was admitted to the hospital for a third time because of persistent hepatosplenomegaly and leucocytosis. Upon admission, his haemoglobin was 13.5 g/100 ml; PCV 43.1%,

WBC 52 000/μl; differential count: 39% segmented PMN; 38% bands; 10% metamyelocytes; 1% myelocytes; 1% promyelocytes; 1% blasts; 4% lymphocytes; 6% monocytes; platelet count 271 000/μl. Bone marrow aspiration revealed myeloid and megakaryocytic hyperplasia with scanty representation of the red cell series. Iron stain revealed adequate stores.

TABLE I. Serum vitamin B_{12}, $UB_{12}BC$, and B_{12} binders

Date	*Vitamin B_{12} (pg/ml)*	*$UB_{12}BC$ (pg/ml)*	*B_{12} binders (%)*	
			α	β
25 May 1964	163			
15 June 1964	234			
19 April 1968*	151	2692	30.6	69.4
26 April 1968	606	2564	27.6	72.4
15 July 1968	382	4198	47.6	52.4
26 August 1968	701	3080	53.6	46.4
18 November 1968	662	4002	44.0	56.0
3 February 1969	620	3936	72.7	27.3
12 May 1969	441	3515	56.5	43.5
24 June 1969	406	3222	40.4	59.6
8 October 1969	318	2886	37.7	62.3
18 November 1969	309	2852	43.9	56.1
19 December 1969	498	2816	42.0	58.0
5 March 1970	200	3172	38.2	61.9
10 June 1970	207	2806	35.7	64.3
24 July 1970	364	2543		
17 August 1970	67	3736	51	49
31 August 1970	240	4418	59	41
28 September 1970	443	4513	56	44
2 November 1970	270	3076	40	60
Normal	200–900	980–1756	15–25	75–85

* Drawn in Emergency Room, before giving 1000 μg vitamin B_{12} to patient. Haemoglobin at the time was 5.5 g/100 ml.

Serum vitamin B_{12} was 382 pg/ml; folate 12.5 ng/ml; unsaturated vitamin B_{12} binding capacity ($UB_{12}BC$) 4198 pg/ml, with an α/β distribution of 48%/52% respectively. Blocking antibody to intrinsic factor was detected in the serum. Serum iron was 80μg/100 ml; unsaturated iron binding capacity 254 μg/100 ml. Serum haptoglobin was 200 mg/100 ml; uric acid 7.2 mg/100 ml; neutrophil alkaline phosphatase score 21. Chromosomal studies revealed the presence of a Philadelphia chromosome (chromosomal studies performed at Dr K. Hirschhorn's laboratory at The Mount Sinai Hospital, New York).

While in the hospital, the patient's leucocytosis was stable in the 40 000–50 000/μl range, and his hepatosplenomegaly disappeared. It was felt that further vitamin B_{12} therapy could result in an increase in the WBC count and the patient was therefore discharged on 19 July 1968, on no therapy for his haematologic conditions, to be followed at the Hematology Clinic.

During the period the patient was followed in the Hematology Clinic, he had consistently

low neutrophil alkaline phosphatase determinations, with values of 35 (18 July 1968), 31 (29 July 1968), 20 (13 August 1968), 26 (27 August 1968), 14 (4 February 1969) and 8 (12 May 1969). Those values above 25 never showed more than 1+ staining in the neutrophils. His $UB_{12}BC$ and α/β distribution remained abnormal (Table 1). Serum vitamin B_{12} levels (Table 1) were normal in spite of an increase in the red cell mean corpuscular volume, marked hypersegmentation of the PMN leukocytes and evidence of deranged DNA synthesis (Fig 1). A radioactive vitamin B_{12} clearance showed delayed clearance of the vitamin (Fig 2), as seen in patients with CML. Five μg of vitamin B_{12} were administered parenterally on 19 August 1968. The serum vitamin B_{12} level on 26 August 1968 was 701 pg/ml (Table 1), slowly decreasing over a 2 yr period to 207 pg/ml on 10 June 1970.

The patient's condition has remained stable throughout this period; there has been no evidence of peripheral neuropathies. His serum vitamin B_{12} has dropped to the 150–200 pg/ml range, on no therapy. His haemoglobin level has also been stable between 10.5 and 11.5 g/100 ml on multiple determinations.

MATERIALS AND METHODS

Serum vitamin B_{12} was assayed by the coated charcoal method (Lau *et al*, 1965), as was the unsaturated vitamin B_{12} binding capacity ($UB_{12}BC$) and the serum assay for intrinsic factor antibodies (Gottlieb *et al*, 1965). Fractionation of the vitamin B_{12} binding proteins was done by DEAE cellulose chromatography on a small column (Retief *et al*, 1967). Serum folate was determined microbiologically with *L. casei* (Herbert, 1966).

For studies of DNA synthesis, heparinized blood was obtained from the patient and the cells were harvested and cultured as previously reported for bone marrow cells (Metz *et al*, 1968). The plasma was removed and the cells washed twice with 10 ml of Tris-buffered (pH 7.2) Hanks' balanced salt solution (HBSS). The cells were finally suspended in autologous serum and Tris-HBSS (1:2 v/v), to give a final concentration of 10 000–20 000 nucleated red cells/μl. Three-tenths ml aliquots of this suspension were dispensed into 10 ml Vacutainer® tubes (No. 3200, Becton, Dickison & Co., Rutherford, N.J.), all experiments being performed in triplicate. Folic acid (PteGlu) (Sigma Chemical Co., St Louis, Mo.), folinic acid (CF) (racemic mixture obtained from Lederle Laboratories), and vitamin B_{12} (Squibb Laboratories) were added in volumes of 0.1 ml to give final concentrations of 50 μg/ml, 50 μg/ml and 1 μg/ml, respectively. Deoxyuridine (dU) (Sigma Chemical Co.), dissolved in Tris-HBSS was also added in 0.1 ml aliquots to a final concentration of 10^{-1} μmole/ml. Tris-HBSS was then added to complete a volume of 0.9 ml in all tubes. Preincubation for 15 min at room temperature was carried out when PteGlu, CF or vitamin B_{12} were utilized, prior to the addition of the dU. Preincubation with dU lasted for 1 hr and was also carried out at room temperature. After preincubating with dU, each tube received 1 μCi of tritiated thymidine (obtained as [^{3}H methyl]thymidine from Tracerlab, Waltham, Mass.; specific activity 10.5 Ci/mM ([^{3}H]TdR)). A 3 hr incubation, at 37°C, in a Dubnoff metabolic shaker (60 cycles/min) was then carried out, after which extraction of DNA from the cell suspension was performed as described previously (Cooper & Rubin, 1965; Metz *et al*, 1968). Radioactivity was measured in a Picker Nuclear Liquimat and results expressed as percent suppression of [^{3}H]TdR incorporation into DNA. Similar studies were done with cells obtained

from another patient with CML, without evidence of vitamin B_{12} or folate deficiencies. Results presented (Fig 1) are the average of three different experiments.

An intravenous vitamin B_{12} disappearance study was performed by injecting 0.45 μg sterile [^{57}Co]vitamin B_{12} (Squibb laboratories; specific activity 11 μCi/μg) into a forearm vein and obtaining venous blood samples from the opposite arm at various intervals as described by Brody *et al* (1960), modified from Mollin *et al* (1956).

RESULTS

Serum Vitamin B_{12} Levels, $UB_{12}BC$ and Vitamin B_{12} Binding Proteins

As shown in Table I, the serum vitamin B_{12} level in the patient has remained normal in spite of evidence of megaloblastosis by morphologic and biochemical (Fig 1) criteria. The unsaturated vitamin B_{12} binding capacity ($UB_{12}BC$) has also been persistently elevated as has been the percentage of alpha-binding protein.

DNA Synthesis Studies

Fig 1 shows the results obtained when the patient's peripheral blood cells were studied. Similar studies performed with peripheral blood obtained from another patient with CML (without evidence of vitamin B_{12} or folate deficiency), are included as controls.

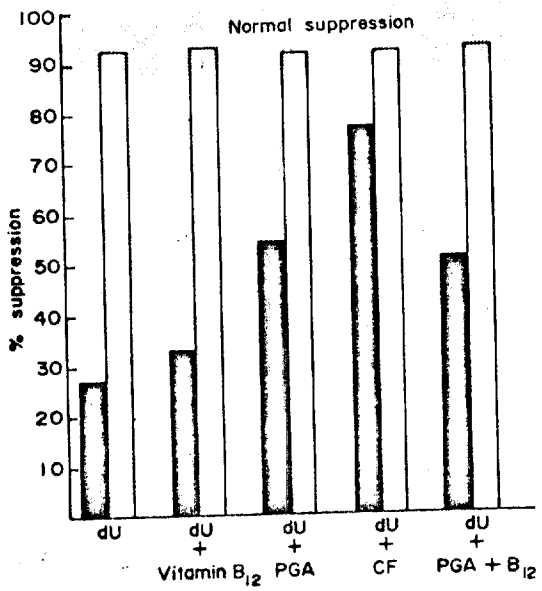

FIG 1. Percentage suppression of [^{3}H]TdR incorporation into DNA by a 1 hr preincubation with 10^{-1} μmole/ml deoxyuridine. Solid columns, patient (CML+PA); open columns, control (CML).

When marrow (or immature peripheral blood) cells are preincubated with dU, at concentrations of 10^{-1} μmole/ml, for 1 hr, prior to the addition of [^{3}H]TdR, the incorporation of the latter is normally suppressed by approximately 90% (Metz *et al*, 1968). In our hands, this system provides a sensitive tool for the detection of deranged folate and/or vitamin B_{12}

metabolism, even when the serum folate (Corcino *et al*, 1970) or vitamin B_{12} (as this case represents) is normal.

The abnormal suppression of [^{3}H]TdR incorporation present in the peripheral blood cells of the patient, as compared to a control (27% *v* 92%), is clearly shown in Fig 1. Preincubation with PteGlu and CF results in increased suppression of [^{3}H]TdR incorporation. Preincubation with vitamin B_{12}, on the other hand, had less effect. Similar results have been reported from this laboratory for bone marrow cells obtained from patients with PA (Metz *et al*, 1968).

Intravenous [^{57}Co]Vitamin B_{12} Disappearance Study

Fig 2 shows the results of the clearance of [^{57}Co]vitamin B_{12} by the patient as compared to normals, patients with PA in relapse, and patients with CML. As shown, the disappearance of the injected [^{57}Co]vitamin B_{12} was slower than that reported for patients with CML (Brody *et al*, 1960).

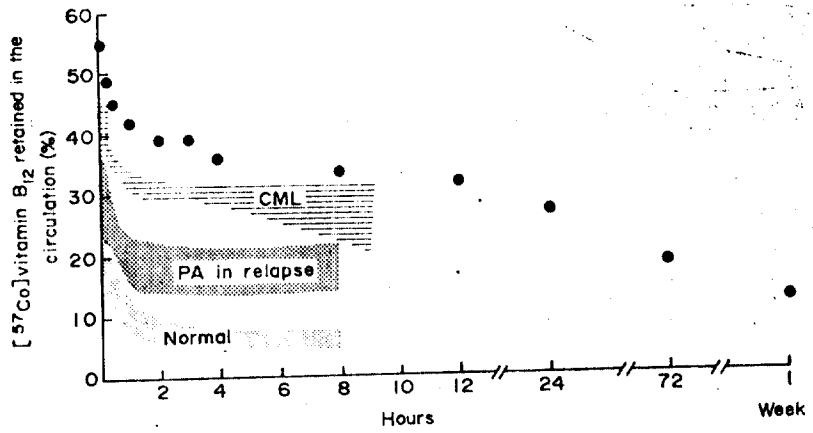

FIG 2. [^{57}Co]vitamin B_{12} disappearance curve.

DISCUSSION

The coexistence of myasthenia gravis (MG), PA and CML in the patient described here, seems to be coincidental. Simpson (1964) noted only one case of PA among 407 patients with MG in a London series, and Osserman (1965) has reported two cases of PA in a series of 687 patients with MG seen in The Mount Sinai Hospital. Thus, although both disorders may be related to autoimmune phenomena, there does not appear to be an increased frequency of clinical PA in patients with MG.

The association of PA and CML is also rare; review of the literature reveals seven case reports of this combination (Woolley, 1944; Sterne *et al*, 1941; Tawast & Siurala, 1956; Blackburn, 1957; Hitzenberger, 1958; Mosbech, 1959; Britt & Rose, 1966), which seems to occur in a purely coincidental manner. Patients with PA do not have an increased incidence of CML.

All three diagnoses are well substantiated in our patient. MG is substantiated by the clinical

presentation, results of muscle biopsy and the dramatic response to Mestinon. The diagnosis of PA is established by the presence of histamine-fast achlorhydria, subnormal Glass test which corrects with intrinsic factor, and the presence of circulating blocking antibody to intrinsic factor. A diagnosis of CML is supported by the persistent leucocytosis with immature myeloid cells in the peripheral blood, presence of a Philadelphia chromosome, and the elevated $UB_{12}BC$ and increased unsaturated α $B_{12}BC$. The patient's neutrophil alkaline phosphatase score has always been subnormal, although not the zero value usually seen in patients with CML (Koler *et al*, 1958). A series of CML patients with normal neutrophil alkaline phosphatase score has been reported recently (Krauss, 1968).

Endogenous serum vitamin B_{12} circulates bound to a glycoprotein with α electrophoretic motility (Pitney *et al*, 1954) and an approximate molecular weight of 115 000 (Hom & Olesen, 1967). Fractionation of serum vitamin B_{12} binders by paper electrophoresis led to the discovery of a second serum binder (Pitney *et al*, 1954) which does not normally contain measurable amounts of vitamin B_{12} but which is capable of binding approximately 80% of the vitamin B_{12} added *in vitro* (Retief *et al*, 1967). The physiological role of the two serum vitamin B_{12} binding proteins has been studied in various systems (Finkler & Hall, 1967; Retief *et al*, 1967). The physiological role of this binder remains uncertain.

It has been known for several years that patients with CML have high serum levels of vitamin B_{12} (Beard *et al*, 1954; Mollin & Ross, 1955) and unsaturated α binding protein (Beard *et al*, 1954). Similar but less striking high levels in other myeloproliferative disorders have also been reported (Gilbert *et al*, 1969). The diagnostic and prognostic values of measurement of these parameters related to vitamin B_{12} metabolism, in various conditions, have been reviewed recently (Herbert, 1968).

The patient reported in this communication provides several interesting observations which may help shed some light on the metabolism of vitamin B_{12} in the myeloproliferative syndromes. When vitamin B_{12} deficiency coexists with CML, the serum vitamin B_{12} levels may be misleading since they may be normal or elevated even though the intracellular stores of the vitamin may be low. The presence of deranged DNA synthesis in the peripheral blood cells of the present patient, even though his serum vitamin B_{12} level was within the normal range at the time of the study, supports this concept. Thus, even though the serum content of vitamin B_{12} is normal, delivery of the vitamin to tissues is deficient, presumably because it is bound to a protein (α-binder) that lacks adequate delivery capacity. In this sense, the vitamin is 'trapped' in the circulating blood, and can be considered 'metabolically dead'. The further possibility exists that the patient's cells may have a defective mechanism for uptake of vitamin B_{12}, as seems to be the case for vitamin B_{12} deficient cells in the absence of coexisting CML (Retief *et al*, 1966). Such a mechanism may be further exaggerated as the CML cell becomes more vitamin B_{12} deficient.

The intravenous vitamin B_{12} disappearance study provides further support for the concept of deficient delivery of vitamin B_{12} to cells in patients with CML. When vitamin B_{12} is injected intravenously into patients with CML, it disappears from the circulation at a slower rate than in normal subjects (Fig 2), as previously reported by others (Mollin *et al*, 1956; Brody *et al*, 1960) suggesting that it is bound to a protein that does not deliver the vitamin to cells. When the sera of patients with CML is fractionated in DEAE cellulose after the *in vitro* addition of vitamin B_{12}, the normal α/β ratio of 1/4 is reversed. Patients with PA in relapse

also present delayed clearance of intravenously injected vitamin B_{12}. This has been attributed to an increase in the unsaturated α binder present in their sera (Schiffer *et al*, 1966), plus the aforementioned defective vitamin B_{12} uptake by vitamin B_{12} deficient cells.

Frank leucocytosis was not recorded in the present patient until after he was given an injection of vitamin B_{12}. It is possible that his elevated unsaturated α binder rendered his low (for a CML patient) serum vitamin B_{12} metabolically unavailable to his CML cells, and thus constituted a 'built-in' mechanism for suppressing his leucocytosis by withholding vitamin B_{12} necessary for DNA synthesis. The injected mass of vitamin B_{12} was far in excess of the amount necessary to saturate his $UB_{12}BC$, and some of the excess could presumably enter the CML cells by diffusion. With this in mind, we withheld vitamin B_{12} therapy from the patient since the 5 μg he received in August 1968. His peripheral blood white count remained stable in the range of 40 000–60 000 WBC/μl for 2 yr. We intended to withhold vitamin B_{12} for as long as possible, watching carefully for any neurological damage.

On 17 August 1970 the patient was given an injection of 100 μg of vitamin B_{12} by a physician who was not aware that therapy had been deliberately withheld. On that date, the patient had a haemoglobin of 8.3 g/100 ml, haematocrit 31%, red cell count 2.3×10^6/μl; platelet count 88 000/μl; WBC 52 500/μl; the spleen was palpable 16 cm below the left costal margin in the midclavicular line at rest (as compared to 10 cm on 24 July 1970) and the patient felt weak. On 31 August 1970 his haemoglobin was 9.2 g/100 ml, haematocrit 33.5%; platelet count 230 000 μl; WBC 129 000/μl, his spleen size had receded to that of 24 July 1970 and he felt much stronger. He was given another 100 μg of vitamin B_{12} by injection. On 28 September 1970 his haemoglobin was 9.2 g/100 ml, haematocrit 35%; platelet count 130 000/μl; WBC 61 000/μl. The WBC values recorded are all total nucleated cell counts. (The WBC figures would be reduced by about 15% when corrected for the 15–17 nucleated red cells present per 100 white cells.)

Table I presents the levels of serum vitamin B_{12} and unsaturated vitamin B_{12} binding capacity in association with this period of vitamin B_{12} therapy. The rise in unsaturated B_{12} binding capacity after B_{12} therapy would suggest an increase in the total blood granulocyte pool (Chikkappa *et al*, 1971). It remains to be seen whether this rise in unsaturated vitamin B_{12} binding capacity associated with vitamin B_{12} therapy presages the patient's leukaemia entering the acute phase.

Some similiar phenomena have been observed in the B_{12} metabolism of a patient with coexistent polycythaemia vera and pernicious anaemia (Sage 1969).

Note added in proof. In January 1971 the patient was again noted to have anaemia, thrombopenia, splenomegaly a lowered WBC (25 300/μl), weakness, and a serum B_{12} level of 157 pg/ml. A single injection of 5 μg of vitamin B_{12} again reverted these parameters to their prior status.

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The Effect of Deconjugated and Conjugated Bile Salts on the Intestinal Uptake of Radio-vitamin B_{12} *in Vitro* and *in Vivo*[1] (37238)

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Vitamin B_{12} and bile acids have their active transport mechanisms located in the terminal ileum both in man and in the guinea pig. It seemed reasonable to explore whether these transport mechanisms were separate or interrelated; and whether the presence of bile salts affected the absorption of vitamin B_{12}. This may have clinical significance in the "blind loop syndrome" where it has been shown that bacterial overgrowth in the small intestine deconjugates bile acids and inhibits vitamin B_{12} absorption.

Methods. In vitro, the guinea pig intestinal mucosal homogenate (GPIMH) assay described by Castro-Curel and Glass (1) was used. Gastric juice (GJ) was collected from a normal volunteer by performing a maximal histamine stimulation test. The 30 and 60 min specimens were pooled, brought to pH 10 with 1.0 *M* NaOH, and then to pH 7.0 with 1.0 *M* HCl. The neutralized GJ was then strained through gauze, divided into 2 ml aliquots and frozen. Each assay was performed in duplicate in the following manner: to a 15 ml test tube containing 5 ml of Krebs–Henseleit bicarbonate glucose medium, the following were added: (a) 1 ml of ^{57}Co-B_{12} containing 2500 pg vitamin B_{12} (B_{12}), the specific activity of which provided 4–6 cpm/pg; (b) 1 ml suspension of GPIMH in normal saline containing 12 mg GPIMH; (c) 0.025 ml of GJ diluted to 1 ml with normal saline; and (d) 1 ml of the various bile acids in normal saline in a concentration of 1000 μg/ml. The bile acids (BA) were converted to their sodium salts by adding 0.1 *M* NaOH in dropwise fashion until all of the bile acid was seen to go into solution. We tested the two conjugated bile acids, glycocholic acid (GC) and glycodeoxycholic acid (GDC) and their deconjugated counterparts cholic acid (CA) and deoxycholic acid (DC). In test tubes which did not contain GJ or BA, 1 ml of normal saline was substituted instead. The reagents were mixed in different sequences as follows:

1. B_{12} + GPIMH;
2. B_{12} + BA + GPIMH;
3. B_{12} + GJ + GPIMH;
4. GJ + B_{12} + BA + GPIMH;
5. B_{12} + BA + GJ + GPIMH;
6. BA + GJ + B_{12} + GPIMH;
7. GPIMH + BA + GJ + B_{12}.

Each of the reagents was well mixed into the incubation solution prior to the addition of the next reagent. Sequences 1 and 2 aimed at studying the effect of the respective bile acids on the nonintrinsic factor-mediated uptake of radio-B_{12}. Sequence 3 acted as a control showing the increased uptake of B_{12} by the GPIMH with intrinsic factor (IF)-containing GJ. Sequences 4 through 7 aimed at studying the effect of the different bile salts on the various phases of the IF-mediated uptake of radio-B_{12} by the GPIMH.

The mixtures were incubated at 5° for 1 hr as described by England, Ashworth and Taylor (2) rather than at 37°. This was found to lower the non-IF-mediated uptake, but had no effect on IF-mediated uptake thereby increasing the difference between the two 7- to 10-fold as opposed to incubating at 37° which showed a difference of 2- to 3-fold.

Following incubation, the GPIMH was

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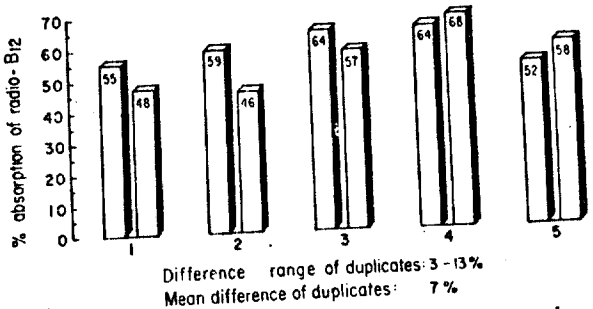

FIG. 1. The reproducibility of the double label hepatic uptake test performed twice in five consecutive normal adult volunteers.

centrifuged, washed, and counted in a heavily shielded well counter as previously described (1).

In vivo, the double label hepatic uptake test (DLHU) of Weisberg and Glass (3) was used. This test permits the quantitative measurement of radio vitamin B_{12} absorption. The test was performed by measuring the hepatic uptake 14 days after the oral administration of 1.0 μg ^{60}Co-B_{12} (0.51 μCi/μg) and comparing it with that of a simultaneously administered intravenous tracer dose (0.05 μg) of ^{57}Co-B_{12} (10 μCi/μg) after correcting for the different counting characteristics of the two cobalt isotopes. The reproducibility of the test in the same individual was determined by performing the test twice at 2 wk intervals. Five consecutive normal adult volunteers were studied. The mean of the difference in B_{12} absorption values of the duplicates was 7% and the range of the difference of the duplicates was 3–13% (Fig. 1). The test was then performed on two normal volunteers before and after oral administration of cholic acid orally, 250 mg three times daily for 3 days. Radio-B_{12} was given on Day 2 and the hepatic uptake was counted 14 days later.

Results. In vitro, GC and GDC had no effect on the non-IF- and IF-mediated uptake of B_{12} by the GPIMH as is shown in Figs. 2 and 3. DC had no effect on non-IF-mediated uptake of B_{12} but caused a slight decrease in the IF-mediated uptake when added in the sequences 4, 5, and 7 (see Methods) shown in Fig. 4. This decrease was not statistically significant. CA, however, produced a 50–55% decrease in both non-IF- and IF-mediated uptake of B_{12} regardless of the sequence in which it was added (Fig. 5).

In vivo, the effect of CA on the absorption of B_{12} in two normal individuals, as measured by the DLHU test, was studied. The first individual was given CA as previously described during the first measurement and none during the second. The sequence was reversed in the second patient. In the first patient the absorption of ^{60}Co-B_{12} was decreased from 48 to 15% while on CA, and in the second patient it was decreased from 66 to 40% (Fig. 6).

Discussion. These studies indicate that cholic acid, the unconjugated bile acid, inhibits the uptake of radio-B_{12} by the GPI MH and decreases the absorption of radio-B_{12} in 2 normal volunteers. The fact that it inhibited the uptake of radio-B_{12} by the GPI MH regardless of the sequence in which it was added suggests it produces its effect by a direct action on the ileal mucosa rather than by interfering with the formation of the intrinsic factor-vitamin B_{12} complex.

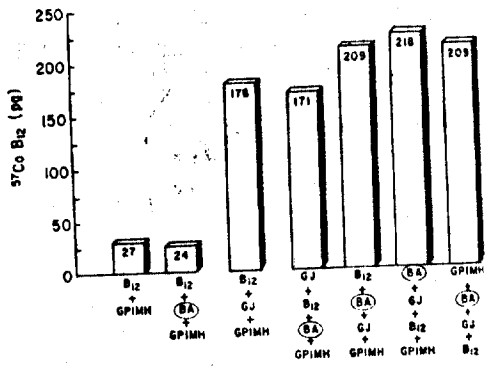

FIG. 2. The effect of glycocholic acid on the uptake of ^{57}Co-B_{12} by the GPIMH.

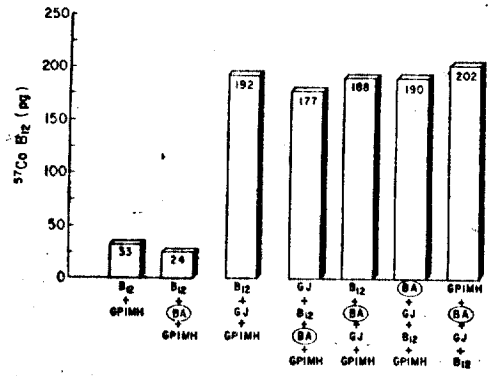

FIG. 3. The effect of glycodeoxycholic acid on the uptake of ^{57}Co-B_{12} by the GPIMH.

Low-Beer, Schneider and Dobbins (4) have described significant morphological changes in the intestine of the guinea pig and hamster following *in vitro* incubation and *in vivo* perfusion with sodium cholate. Unconjugated bile salts have also been shown to inhibit several intestinal mechanisms concerned with the transport of water-soluble nutrients by the rat jejunum *in vitro* which was not the case with conjugated bile salts (5). Thus, it would seem reasonable to assume that the decrease in absorption of B_{12} produced by CA is the result of a direct toxic effect on the ileal mucosa.

A number of drugs have been shown to interfere with the absorption of vitamin B_{12} by direct injury to the ileal mucosa. Jacobsen, Chodos and Faloon (6) demonstrated that the administration of neomycin sulfate for 6 to 9 days reduced the urinary excretion of ^{60}Co-B_{12} by 20 to 60% in four of six subjects. Dobbins, Herreo and Mansback (7) subsequently demonstrated abnormal small intestinal morphology in neomycin-induced malabsorption. Lindenbaum and Lieber (8) demonstrated impaired absorption of vitamin B_{12} in subjects given 158 to 253 g of ethanol daily for 2 wk along with adequate protein and vitamin intake. This impaired absorption was accompanied by striking ultrastructural changes in an ileal biopsy obtained from one subject.

Our findings further clarify the possible mechanism of B_{12} malabsorption in "the blind loop syndrome." Schjönsby *et al.* (9) have demonstrated that certain enteric organisms such as Bacteroides and Enterobacteria are capable of partly binding the IF-B_{12} complex *in vitro*. They showed that in patients with bacterial contamination of the small bowel following a small oral dose of IF-bound ^{57}Co-B_{12}, a considerable amount of radioactivity (43–72%) is recoverable from ileal aspirates. Less than 11% could be recovered from control subjects. This was taken to indicate that B_{12} malabsorption occurred in cases of the blind loop syndrome because of the bacterial binding of the IF-B_{12} complex. In light of the present information, it could equally be concluded that the increased radioactivity in the ileal aspirates and malabsorption of B_{12} may be the result of bacterial deconjugation of bile salts and their subsequent inhibition of B_{12} absorption by their direct toxic effect on the ileal mucosa.

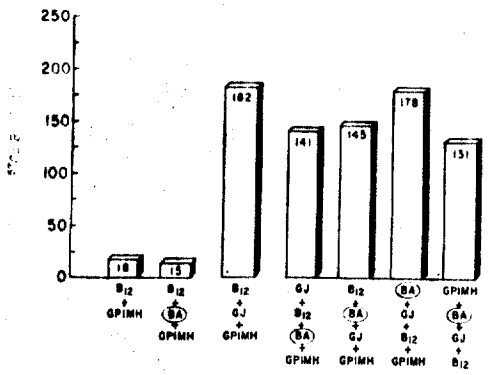

FIG. 4. The effect of deoxycholic acid on the uptake of ^{57}Co-B_{12} by the GPIMH.

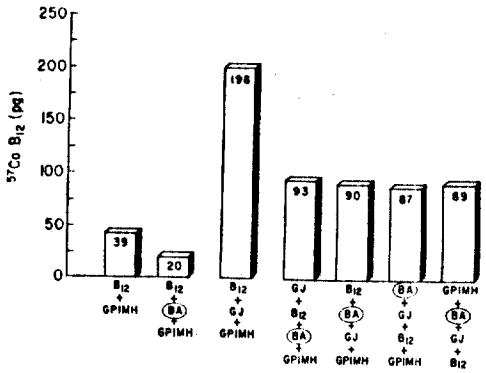

FIG. 5. The effect of cholic acid on the uptake of ^{57}Co-B_{12} by the GPIMH.

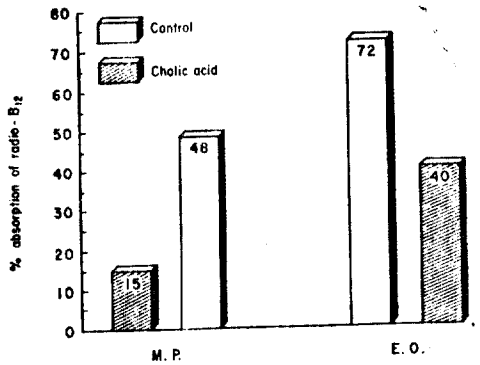

FIG. 6. The effect of cholic acid on the absorption of radio-B_{12} in normal volunteers as measured by the double label hepatic uptake test.

Summary. Deconjugated cholic acid decreased the intestinal uptake of radio-B_{12} by the guinea pig intestinal mucosal homogenate by 50 to 55%. It also decreased the absorption of radio-B_{12} in 2 normal adult volunteers as measured by the double label hepatic uptake test. Cholic acid was felt to produce this decrease by a direct toxic effect on the ileal mucosa. Deoxycholic acid was found to produce a slight but not statistically significant decrease in the uptake of radio-B_{12} by the guinea pig intestinal mucosal homogenate. Glycocholic acid, and glycodeoxycholic acid had no statistically significant effect on radio-B_{12} uptake by the guinea pig intestinal mucosal homogenate.

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Vitamin B_{12}
Vitamin Supplements
Optimum Versus Minimum Requirement
Ribonucleic Acid
Behavioral Measures

Task Specificity of Effects
Strain of rat
Age of rat
Dose level
Duration of treatment

High Levels of Vitamin B_{12} Versus Minimum Requirement: Biological and Behavioral Differences

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Summary: We had previously found that in Long-Evans rats maintained on high levels of vitamin B_{12} (and compared with rats maintained on enough vitamin B_{12} to exceed the minimal nutritional requirement) there was an improvement in performance of a simple avoidance task ($p < .02$) and of a pattern discrimination ($p < .001$) as well as an increase in the concentration of RNA in the nucleoli of neurons in the cerebellum and spinal cord.

In the present three experiments using a total of 84 laboratory rats as subjects, the relationship between high levels of vitamin B_{12} and performance of various tasks was further examined. Variations in strain of rat, dose level, length of injection period and age of rat at onset of vitamin B_{12} treatment were investigated. In adult rats of both the Long-Evans ($p < .05$) and Sprague-Dawley ($p < .06$) strains high levels of vitamin B_{12} improved pattern discrimination relative to the level of performance shown by control animals (which were again maintained on enough vitamin B_{12} to exceed the minimal nutritional requirement). However, there were certain detrimental effects in other situations on Sprague-Dawley rats ($p < .05$) and no significant effects on the performance of

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younger Long-Evans rats. The facilitation of pattern discrimination in Sprague-Dawley rats occurred over a wide range of high doses and a wide weight-age range. The effect was found with a 20-day but not with a 2-day pretest injection period. The results are interpreted as confirming the existence of a relationship between high levels of vitamin B_{12} and some aspects of cognitive function, and as suggesting certain remaining problems in the study of optimum versus minimum nutritional requirements.

Many recent studies of the biological basis of memory [10] have been concerned with the role of intracellular neural ribonucleic acid (RNA). A report that administration of high levels of vitamin B_{12} increases RNA levels in spinal neurons of young rats [1] led to further examination in our laboratories of some of the biochemical and behavioral effects of vitamin B_{12}. Enesco [7] found that in Long-Evans rats 20 days of injections of 0.40 µg of vitamin B_{12} produced an increase in the concentration of RNA in the nucleoli of neurons in the cerebellum and spinal cord. Corson [3] showed that vitamin B_{12} injections (using the same dose and injection routine as that of Enesco) improved the performance of Long-Evans rats in simple avoidance and in pattern discrimination situations with shock avoidance as the motivation. Soon after we made these observations, Pauling [11] published a theoretical discussion which provided a new perspective for our work. His basic notion was that biochemical abnormalities such as «cerebral avitaminosis» could be at the basis of certain forms of mental illness. Furthermore he suggested that nutritional interventions including massive doses of vitamins could be expected to be of benefit to some people who were already receiving more than the minimum daily requirement of all substances assumed to be dietary necessities. Since our control subjects were maintained on enough vitamin B_{12} to exceed the minimum daily requirement our work can be seen as providing a comparative evaluation of some of Pauling's ideas.

The present behavioral studies were done to examine the roles of the following variables in the vitamin B_{12} effect: strain of rat, dose level, length of injection period and age of rat at onset of vitamin B_{12} treatment.

General Materials and Methods

All subjects were obtained from the Canadian Breeding Laboratories. They were housed 1 per cage in 9½" x 7" cages. A 12 hour light, 12 hour dark cycle was imposed. Rats were maintained on *ad lib.* food and water. The food was Purina Rat Chow which contained .02 µg of vitamin B_{12} per g. (This figure was given by the Purina Company and its accuracy and variability are unknown to us.) Since each of the adult rats (used in all but Experiment 3) consumed about 15 g of chow

per day, they received a daily total of 0.8 μg of vitamin B_{12} in their food. The estimated daily vitamin B_{12} requirement is 0.025 to 0.1 μg [13]; therefore, all rats received at least 3 times the estimated daily requirement of vitamin B_{12}.

The following facts are presented as evidence that the control rats were indeed receiving enough vitamin B_{12} to exceed the minimal nutritional requirement:

(a) None of the rats in the present series of experiments showed deficiency signs such as lowered food consumption or abnormal growth rate [13].

(b) The growth rate during the experimental period was identical for experimental and control animals.

(c) Rats fed with the diet received by our control subjects and used in other experiments have not produced deformed offspring or shown slow wound healing, both of which are signs of vitamin B_{12} deficiency [13].

All animals were handled for 2 days (5 min per rat per day) prior to the start of the injection period. Vitamin B_{12} was obtained from British Drug House Limited and was placed in saline solution in an appropriate concentration for the injection level given in each experiment. Injections were given between 2 and 4 hours before the end of the light period. All injections were intraperitoneal and the control rats were given an amount of saline equal to the volume of substance administered to the experimental rats. Testing was done between 1 hour after the start of the light period and 2 hours before the end of the light period. Groups were evenly distributed with regard to the time of day that their testing took place and the order in which the injections were given. All experiments were run blind in that the person testing the subjects had no knowledge of group membership.

Experiment 1

In the first experiment we examined the effect of 0.40 μg vitamin B_{12} injections on the behavior of animals of a strain of rat (Sprague-Dawley) different from that (Long-Evans) used by Corson [3]. Except for the difference in strain this study was identical in all aspects to the earlier study.

Method

Subjects were 22 male Sprague-Dawley rats, weighing an average of 210 g (ranging from 190 to 230 g) at the start of the injection period. There were 12 experimental and 10 control rats and the injections were given each day for 20 days prior to the beginning of testing and were continued throughout the testing period. The animals weighed an average of 270 g at the start of testing (ranging from 250 to 300 g).

The electrified two-choice discrimination box previously used by Corson [3, 4] was used in this study. The apparatus and technique were adapted from Thompson and Bryant [14]. This apparatus was divided into 5 sections as follows: start area, choice area, 2 threshold areas and goal area. All but the goal area had grid floors which could be electrified by the experimenter. On the first training day the subjects learned to go quickly from the start area to the goal through either door to avoid shock. The start grid was electrified 5 seconds after opening of a door which separated the start area from the rest of the apparatus and all grids were electrified 25 seconds later. Having reached a criterion of 3 consecutive trials completed in less than 30 seconds in this pretraining phase, they next learned to enter the goal by crossing the threshold area on one side only (spatial discrimination) to the criterion of either 5 consecutive errorless runs or 6 errorless runs in 7 trials. During this latter phase the incorrect door was locked and its threshold area was electrified. On the second day the animals learned to enter the goal by crossing the threshold area

opposite to that which had been correct on the first day (spatial reversal) and the same criterion was used.

The rats were next required to learn a discrimination between horizontal and vertical black and white stripes which were painted on cards placed on the doors to the goal area. These cards were alternated in a prearranged random order [8] from one door to the other, with the positive card always placed on the unlocked door. The threshold area in front of the locked door was always electrified. The rats were given 15 trials per day for 4 days with the horizontal stripes as the positive stimulus (pattern discrimination learning).

Results and Discussion

The results of this experiment with Sprague-Dawley rats show the same order of difference in pattern discrimination that had been found in the previous study with Long-Evans rats (see Table I). The experimental animals made fewer errors than the control animals two-tailed t-test $p < .05$). However, the results show that the experimental animals took more time to get out of the start box in the pre-training phase ($p < .01$), took more trials to make their first avoidance of shock in the start box ($p < .05$) and took more trials to reach criterion in the spatial learning phase ($p < .02$). The experimental animals were also slightly slower in getting out of the start box in the spatial learning phase ($p < .10$) and took slightly fewer trials to reach criterion in the spatial reversal phase ($p < .10$).

The effect on pattern discrimination is the only clear similarity between the present study and that of Corson [3]. Otherwise the results with the Sprague-Dawley rats of the present study (showing some detrimental effects) are much more complex than were obtained with Long-Evans rats. However, since these were separate studies, we are unable to suggest any general conclusions concerning the nature of the differences in effects of vitamin B_{12} on the two strains of rats.

Experiment 2

While the next logical step in this research could be seen as involving a parametric study of the behavioral effects of high levels of vitamin B_{12} on Long-Evans as compared to Sprague-Dawley rats, we decided not to conduct such a study at this time. Part of the basis for this decision was the fact that a large body of previous research had shown differences within a species in the effect of various biological manipulations and more specifically, differences within a species in the needs for certain nutrients had already been demonstrated [16]. Instead we chose to continue with a series of exploratory studies in an attempt to determine the role of some other variables in the production of the behavioral effects of high levels of vitamin B_{12}.

Tab. 1: Effets of Vitamin B_{12} on Performance of Long-Evans and Sprague-Dawley Rats (Weighing 250–300 g) in a 2-choice Discrimination Box

Subjects	Injection	n	Trials to First Avoidance in Start Box	Trials to Criterion			Errors
			Pretraining	Pretraining	Spatial Learning	Spatial Reversal	Pattern Discrimination
			Mean ± SD	Mean ± SD	Mean SD	Mean SD	Mean ± SD
Long-Evans rats from Corson [3]	B_{12}	26	6.1 ± 2.7[c]	9.4 ± 2.2	9.0 ± 2.2	8.9 ± 2.8	14.0 ± 4.1[d]
	Saline	14	9.4 ± 6.0	9.6 ± 4.6	9.6 ± 3.4	10.9 ± 5.3	19.4 ± 4.7
Sprague-Dawley rats from Experiment 1	B_{12}	12	5.5* ± 2.5[b]	8.5 ± 2.3	10.8 ± 3.5[c]	7.1 ± 3.6[a]	27.7 ± 4.2[b]
	Saline	10	3.2* ± 1.2	7.7 ± 2.5	7.5 ± 2.4	9.8 ± 4.9	31.5 ± 4.6

* 3 experimental rats and 1 control rat are not included in this calculation of trials to first avoidance because they failed to avoid shock in the start area during pretraining.

[a] Difference from controls $p < .10$

[b] Difference from controls $p < .05$

[c] Difference from controls $p < .02$

[d] Difference from controls $p < .001$

p values calculated with two-tailed t-test

Tab. II: Effects of Higher Dose of Vitamin B_{12} and of Variations in Injection Period on Performance of Sprague-Dawley Rats (Weighing 350–400 g) in a 2-choice Discrimination Box

Injection	Pre-test Duration of Injections	n	Trials to First Avoidance in Start Box		Trials to Criterion						Errors	
			Pretraining		Pretraining		Spatial Learning		Spatial Reversal		Pattern Discrimination	
			mdn	range	mdn	range	mdn	range	mdn	range	mdn	range
B_{12}	20 days	5	3	2–4[b]	6	6–7[b]	8	6–8	8	6–9	21	19–25[a]
Saline	20 days	4	4	3–6	7	6–12	7	6–8	6.5	6–9	26	22–29
B_{12}	2 days	10	4.5	3–6	7.5	6–8	7.5	6–11	8	6–10	25	21–31
Saline	2 days	4	4	2–7	8	6–9	9	6–13	7	6–9	24	22–30

[a] Difference from controls $p < .06$
[b] Difference from controls $p < .04$
p values calculated with two-tailed Mann-Whitney U-test

The second experiment was carried out to examine both the effect of a higher dose and a shorter injection period on behavior in the same tasks which had been used in Experiment 1. We also used older and heavier subjects in the present experiment.

Method

Subjects were 26 male Sprague-Dawley rats weighing an average of 375 g (ranging from 350 to 400 g) at the start of the testing period. The animals were divided into 4 groups as follows: 5 experimental rats were injected with 2.0 μg vitamin B_{12} for 20 days prior to testing; 4 control rats were injected with the appropriate volume of saline for 20 days prior to testing; 10 experimental rats were injected with 2.0 μg vitamin B_{12} for 2 days prior to testing; 7 control rats were injected with saline for 2 days prior to testing.

Results and Discussion

The results of this experiment (Table II) show that the high-dose, long term injections of vitamin B_{12} produced the same relative difference in pattern discrimination between the experimental and control rats as was found in Experiment 1. The absolute difference between these results and those of the first study appears to be a function of the weight-age difference, since both the experimental and control rats made fewer errors than had their lighter-younger counterparts in the first experiment.

The short term injection of the high dose produced no significant change in behavior. This experiment suggests that the effects of vitamin B_{12} on pattern discrimination do not appear with an injection period as short as 2 days, occur over a wide range of high doses (from 0.4 μg in the first experiment to 2.0 μg in the present experiment), and can be found in Sprague-Dawley rats over a wide weight-age range; however, since we used only two doses and two weights of subjects it must be realized that important complexities may exist between and beyond these extremes.

The rats in the long term injection groups did not show the detrimental effect that was found in Experiment 1. Rather, the experimental rats took slightly fewer trials to make their first avoidance ($p < .04$) and to reach the pretraining criterion ($p < .04$), a finding more in accordance with the previous study of Corson [3]. Further research must be done to determine whether differences in results between this experiment and Experiment 1 are due to the weight-age or the dose difference.

Experiment 3

In the third experiment we examined the effect of beginning vitamin B_{12} injections at an earlier age.

Tab. III: Effects of Vitamin B_{12} on Performance of Young Long-Evans Rats (Weighing 170–210 g) in a 2-choice Discrimination Box

Injection	n	Trials to First Avoidance in Start Box	Trials to Criterion			Errors
		Pretraining	Pretraining	Spatial Learning	Spatial Reversal	Pattern Discrimination
		Mean | SD	Mean | SD	Mean | SD	Mean | SD	Mean | SD
B_{12}	19	7.4 ± 2.2	7.8 ± 2.3	8.4 ± 1.9	7.4 ± 1.5	22.8 ± 5.6
Saline	17	7.5 ± 2.6	7.6 ± 2.6	8.2 ± 2.4	8.3 ± 2.2	24.1 ± 3.3

p values calculated with two-tailed t-test

Method

The rats in this experiment (19 B_{12} injected and 17 saline injected rats) were of the Long-Evans strain. Injections of 0.40 μg of vitamin B_{12} for the experimental animals and an equal volume of saline for the control animals were begun when they were 25 days old (weighing an average of 50 g). The testing began when the rats were 55 days old (weighing an average of 190 g. ranging from 170 to 210 g), which was at least 15 days younger than any of the rats previously used in our studies of vitamin B_{12}. The injection and testing techniques were identical to those used in Experiment 1, except that the animals were injected for 30 days before testing began.

Results and Discussion

The results show no significant effect of vitamin B_{12} on these young animals (see Table III); however, there was a slight difference in pattern discrimination which was in the same direction as we had found in all of our previous experiments. Since the dose of 0.40 μg of vitamin B_{12} is identical to that which we had previously used with positive results [3], it seems that the lack of a significant effect on the pattern discrimination of these rats must be related to age, however, again these were separate studies and no final conclusions can be drawn on this point.

Additional Measurements

After each of the three experiments reported here, we continued injecting the subjects and did further testing with them. We found no differences in learning and extinction of a (shock motivated) pole climbing task, activity in an open field and basal metabolic rate. (The latter two procedures were carried out as crude assessments of changes in excitation or arousal.)

General Discussion and Conclusions

The results of these three experiments show that in adult rats of both the Long-Evans and Sprague-Dawley strains high levels of vitamin B_{12} improved pattern discrimination relative to the level of performance shown by control animals which were maintained on enough vitamin B_{12} to exceed the minimal nutritional requirement. (With regard to the significance or strength of these results it is worth noting that all analyses used 2-tailed tests, even though it can be argued that we could predict a direction of effect on the basis of the large difference ($p < .001$) found in our previous study. If we had predicted and used 1-tail tests for the pattern discrimination the p values would have been $P < .025$ and $P < .03$ respectively. Furthermore, the actual shift in range of performance was striking, with the best experimental subjects being clearly better than the best control

subjects and the worst experimental subjects being not nearly as bad as the worst control subjects).

The facilitation of pattern discrimination in Sprague-Dawley rats occurred at two widely separated doses and with two samples of subjects which were far apart in age and weight. The effect was found with a 20-day pre-test injection period but not with a 2-day period. There were also certain detrimental effects in other situations on Sprague-Dawley subjects, and there was no significant effect on the performance of younger Long-Evans subjects.

The experiments we have done do not permit us to decide whether the primary effects of high levels of vitamin B_{12} are on motivational, visual, cognitive or other processes. However, it seems that any gross effects on motivational processes are ruled out by the presence of the task specificity of the beneficial effects and by the absence of any detectable effects on activity level or BMR. The general question as to whether the effects are on learning or performance cannot be answered without referring to a particular assessment context. In the pattern discrimination it can be argued that the effect is on learning since (a) the effect is found on the error measure and not on the latency measures and (b) both groups reached the level of essentially errorless performance, but with the experimental animals making fewer errors in learning to perform at this level. It is important to note that this task specificity supports the suggestion of Hoff *et al.* [9] that conclusions with regard to dietary adequacy will vary as a function of the methods used to assess nutritional requirements.

Turning to the question of the biological basis of these behavioral effects, one might be tempted to concentrate on the increase in neural RNA produced by injection of vitamin B_{12} and to conclude that there is a direct relationship between this effect and the changes in performance. However, vitamin B_{12} is involved in other processes as well: for example, (a) it is involved in the conversion of methyl malonate CoA in the rat [2], (b) it may detoxify some noxious products of metabolism [17] and (c) it has been implicated in reactions involving carbohydrate, sulfhydryl, lipid and protein metabolism [15]. Since it is possible that these or other presently unknown functions of vitamin B_{12} are responsible for the effects on learning reported here it is clear that these results do not provide any direct support for theories concerning possible relationships between RNA and learning.

In retrospect it is our opinion that workers who study the effects of optimal nutrition as opposed to minimal requirements will have to either operationalize a very narrow set of methods and criteria for assessing any effects of nutritional change or they will have to live for a time with the sort of complexity and

ambiguity seen in our results and conclusions. It is our opinion that the eventual salvation of this effort will be aided by the following: 1) Use of Pauling's theoretical position; 2) Use of behavioral tasks which sample the experimental subject's cognitive behavior at or near the top of his capacity, such as pattern discrimination for the rat; 3) The use of genetic control or analysis of the subject population.

As a first step, the relation of the results of the present experiments to Pauling's theoretical position shows several interesting points of compatibility. For example, the facilitatory effect of high levels of vitamin B_{12} are in accord with his discussion of beneficial effects of massive doses of various nutrients; also the apparent differences in effects between Sprague-Dawley and Long-Evans rats were anticipated by his statements concerning the variability in nutritional requirements in non-homogeneous populations; finally the absence of a significant effect on young Long-Evans rats can be seen as related to his discussion of changes with time in manifestation of the lack of optimum nutrition.

At a more general level it is our opinion that a rigorous examination of Pauling's ideas is called for; this examination should be conducted by workers from a wide variety of disciplines using both animal and human subjects. There are many observations in the literature which suggest that a study of the relationship between nutrition and the function of the nervous system can be expected to have important clinical implications [e. g. 5, 6, 12] and the research strategy of searching for optimum (rather than minimum) requirements of nutrients may be an important asset in the struggle to realize the benefits of these implications.

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Vitamin B12 Absorption and Gastric Antibodies in Rheumatoid Arthritis

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SUMMARY

Vitamin B_{12} absorption below the range of age and sex matched controls occurred in 15 of 52 cases of rheumatoid arthritis. Seven of 10 of these showed increased absorption with hog intrinsic factor. Low B_{12} absorption was more common in the over 65 age group and five of 10 of these patients had parietal cell antibody.

INTRODUCTION

In a preliminary study (Bieder and Wigley, 1964) vitamin B_{12} (B_{12}) deficiency and reduced B_{12} absorption was encountered more frequently than expected from the study of macrocytic anaemia in rheumatoid arthritis by Partridge and Duthie (1963). As B_{12} deficiency may cause neurological and psychiatric symptoms without overt megaloblastic anaemia (Holmes, 1956; Edwin and others, 1965; Strachan and Henderson, 1965), and these changes can be readily overlooked or misinterpreted in rheumatoid arthritis (Bieder and Wigley, 1964) a prospective study to determine the frequency of subnormal B_{12} absorption was considered justified and the findings so far are reported in this paper.

CASE MATERIAL

Case studies were consecutive patients with definite rheumatoid arthritis of whom 42 were women and 10 were men. Controls were healthy volunteers or patients in a medical or orthopaedic ward with no evidence of pernicious anaemia or rheumatoid arthritis. They were selected to be comparable in age and sex with the 52 cases of rheumatoid arthritis. All had normal renal function.

Methods

The Schilling test (Schilling, 1953) was carried out for most of the study with 1.1μc of ^{57}Co B_{12}, but capsules containing 0.5μc were used for the last 12 cases and 15 controls. There was no difference in the range of results (13-52 percent, mean 26 percent) between the two dosage groups, so the results were combined. The percentage of the dose in a 48-hour urine collection was measured, flushing doses of 1000μg. B_{12} being given intramuscularly on both first and second days. Carbachol stimulation was not used. When the urinary excretion of B_{12} was less than 15 percent, the test was repeated when possible with 300mg of hog intrinsic factor (Lederle) given at the same time as the radioactive B_{12}.

The majority of inpatients had a d-xylose absorption test. The five-hour urinary excretion of a 25g dose was measured by the method of Roe and Rice (1948). The excretion in controls was greater than 5g. Normal faecal fat excretion less than 6g/day, estimated by the method of van der Kramer (1958). These tests were carried out under the supervision of a nurse trained in metabolic studies.

Parietal cell antibodies were detected by the immunofluorescent method using human gastric mucosa, (Taylor, Roitt, Doniach, Couchman and Shapland, 1962). B_{12} assays on serum were by the *Lactobacillus leichmanii* method (Matthews, 1962), normal range 250-800pg/ml, and folic acid assays by the *Lactobacillus casei* method (Waters and Mollin, 1961), normal range greater than 2.5ng/ml. The term B_{12} in this paper refers to cyanocobalamine

RESULTS

Schilling Test

Three control cases had B_{12} absorption below the normally accepted range of 13-52 percent. Two of these at 11 percent were normal when repeated and another at 9 percent gave a normal result with intrinsic factor. All rheumatoid arthritis cases below 13 percent are summarised in Table I. There was a clear difference in Schilling test score between controls and rheumatoid cases in the over 65 age group (Table II). The difference was significant for females, and the trend was the same for males. Cases one and two (Table I) had pernicious anaemia.

Seven of 10 rheumatoid arthritis cases retested with hog intrinsic factor showed an increase of at least half of the initial level. Of five patients absorbing 6 percent or less, only one failed to respond to intrinsic factor (Case 3). Case 8 with 7 percent absorption did not respond to intrinsic factor and had an abnormal fat excretion of 10g daily.

A Schilling test score below 5 percent was significantly related to a low haemoglobin or packed cell volume when tested in an analysis of variance, but scores greater than 5 percent showed no significant difference (Table III). The association between serum B_{12} level and Schilling test score was not significant ($P = 0.12$), this would no doubt be stronger where excretions were less than 5 percent, but the numbers in this group were too few, and some of these had possibly been given B_{12}.

Gastric Antibodies

Parietal cell antibodies were found in eight of 13 rheumatoid arthritis cases and one of 23 controls.

TABLE I

FIFTEEN CASES OF RHEUMATOID ARTHRITIS WITH SCHILLING TEST BELOW THE RANGE FOUND IN AGE AND SEX MATCHED CONTROLS

			SCHILLING TEST					
Case	Age	Sex	No IF	Hog IF	D-xylose	Fat Excret. g/Day	Gastric Antibody Fluor.	CFT
1	69	M	4	–	–	4	+	0
2	65	F	4	14	2	2	++	1/8
3	66	F	6	6	–	–	++	0
4	67	M	6	11	6.4	–	0	–
5	72	F	6	13	–	–	++	1/64
6	72	F	6	9	4.6	–	++	trace
7	78	F	6	–	–	1.5	–	–
8	56	F	7	3	7.3	10	0	–
9	72	F	8	20	–	–	–	–
10	80	F	9	10	–	–	0	–
11	88	F	10	–	–	–	–	–
12	66	F	10	29	–	–	–	–
13	84	F	11	–	–	–	–	–
14	90	F	12	–	1.7	4	0	–
15	53	F	12	17	–	3	0	–

TABLE II

SHOWING A SIGNIFICANT ASSOCIATION BETWEEN SCHILLING TEST AND RHEUMATOID ARTHRITIS (RA) AND CONTROLS (C) OVER THE AGE OF 65 YEARS. FISHER'S EXACT METHOD:

	Over 65 Years						Under 65 Years	
Schilling Test	Males		Females		Both Sexes		Both Sexes	
	RA	C	RA	C	RA	C	RA	C
Under 13%	2	1	11	1	13	2	2	1
Over 13%	1	8	11	13	12	21	25	20
Probability	0.127		0.013		0.0012		0.596	

TABLE III

RELATIONSHIP BETWEEN HAEMOGLOBIN, PACKED CELL VOLUME AND SCHILLING TEST

	Mean Packed Cell Volume and SE	Mean Haemoglobin and SE
Schilling % 0– 5	31.1 ± 1.8	8.03 ± 0.74
5–10	38.1 ± 1.3	11.21 ± 0.64
10–15	37.8 ± 1.4	11.73 ± 0.74
Over 15	37.8 ± 0.7	11.78 ± 0.30

The incidence of antibody was signficantly higher, P = 0.0067, where the Schilling test was below 13 percent (Table IV).

Other Findings

Serum folic acid levels below 2.5 nanograms were found in five of 24 rheumatoid arthritis cases. Folic acid levels showed no relation to Schilling test. Nine of 23 cases studied excreted less than 20 percent of 25g of d-xylose in the urine, but the findings showed no correlation with B_{12} absorption or folic acid levels. Schilling test score showed no association with date, season, ESR, rheumatoid factor titres, serum iron levels or severity of disease.

DISCUSSION

This study has demonstrated that the majority of older rheumatoid arthritis cases absorb vitamin B_{12} less efficiently than age and sex-matched controls. The possible explanations for this defect are: lack of

TABLE IV

ASSOCIATION BETWEEN PARIETAL CELL ANTIBODY AND SCHILLING TEST, FISHER'S EXACT METHOD

	Schilling Test	
	< 13%	≥ 13%
Parietal Cell +	5	3
Antibody —	5	34
Probability	0.0067	

intrinsic factor due to atrophic gastritis or gastric atrophy, intrinsic factor antibody, the effect of drugs, or an as yet undisclosed mechanism.

The first of these seems the most probable on present evidence. The response to hog intrinsic factor suggests a lack of endogenous intrinsic factor which could be due to gastric atrophy or atrophic gastritis. It is not necessary to postulate complete atrophy of the mucous membrane as Whiteside and others (1964) have shown reduced uptake of B_{12} in atrophic gastritis and parietal cell antibodies have been found to be associated with atrophic gastritis or gastric atrophy (Coghill, 1965). Wood and others (1964) in a long-term study of histologically proven atrophic gastritis, showed a decline in B_{12} levels with some psychiatric symptoms improving with B_{12} therapy. Nearly half of their patients had parietal cell antibody when tested by the less sensitive complement fixation method. They suggest that atrophic gastritis is a phase in a long continued remitting process which may lead to total atrophy if the patient survives sufficiently long. It is probable that in some cases in the present study, the presence of parietal cell antibody indicates a degree of atrophic gastritis and consequently B_{12} absorption below the normal range in many, but reaching the pernicious anaemia range in a minority. Sufficient histological information is not yet available to clarify this point. The apparent intrinsic factor deficiency could be due to intrinsic factor antibody although this does not seem likely in the cases with uptakes between 13 and 6 percent. This antibody occurs in pernicious anaemia where the uptake is below 6 percent, and its frequency increases with the duration of the disease (Ungar and others, 1967). It is probable that in pernicious anaemia any intrinsic factor produced is neutralised by antibody which prevents binding of vitamin B_{12} impairing absorption. In the present study, most of the Schilling test results are above 5 percent.

In both of these suggested mechanisms, autoimmunity is implicated and this is not surprising in view of the overlap of antibodies in autoimmune diseases and the possibility of the autoimmune origin of rheumatoid arthritis itself (Roitt and Doniach, 1965).

Cases with low B_{12} absorption but no parietal cell antibody in this study suggest that autoimmunity is not the only cause of this defect. In the control patients a regression in B_{12} uptake with age was seen (Couchman, Bieder and Wigley, in preparation). If this is due to reduced intrinsic factor secretion paralleling gastric acidity, then this conforms to the increased incidence of histamine fast achlorhydria with age in normal individuals (Harris, 1963). This regression is more pronounced in rheumatoid arthritis patients over 65 years old.

These patients may also show poor absorption of other substances. Joske and Curnow (1962) found impaired d-xylose absorption in rheumatoid arthritis, and the present study confirms their findings, but shows no relation to B_{12} absorption. The explanation of d-xylose malabsorption is unknown and adequate control data for people of similar age have not been collected. Folic acid also was found to be reduced in a similar proportion of cases to that found by Gough and others (1964) and Deller and others (1966), but anaemia was not definitely shown to be due to this in any of the present cases.

Drug treatment cannot be excluded as a cause of diminished B_{12} absorption although it appears unlikely as the mean B_{12} uptake was lower in patients on no drug treatment than in the drug treatment group. Para-aminosalicylic acid has been shown to reduce B_{12} absorption (Heiniwaara and Palva, 1965), so that it would not be surprising if asprin had a similar effect, being closely related chemically, and this point is being studied further.

Anaemia in rheumatoid arthritis is due to a number of factors and apparently to the disease process itself. This study suggests that defective B_{12} absorption is only of clinical importance in a minority of cases. Possibly a larger group are at risk of developing B_{12} deficiency anaemia especially if their dietary intake of vitamin B_{12} is inadequate, or if they survive long enough to develop gastric atrophy. Their intrinsic factor production, though poor, is possibly sufficient to maintain an adequate B_{12} reserve provided the demand for this vitamin is not increased and diet normal.

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THE IMPORTANCE OF VITAMIN B_{12} FOR PIG FEEDING

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Not all our domestic animals need animal protein. Pigs and poultry, however, react very favourably to a certain amount of animal protein in their feeds, especially in youth. For pigs up to 60 kg. it is sufficient when about half of the protein in the ration is of animal origin (Frens, 1936). Feed mixtures exclusively consisting of plant material are only useful for older and adult pigs.

The fact that young pigs fed on rations containing animal protein displayed an improved rate of growth gave rise at first to the view that the nature and proportions of the amino-acids in the animal proteins supplied the explanation of this phenomenon. The suggestion was that the amino-acid make-up of animal protein was better adapted to the requirements of growing animals. Undoubtedly the amino-acids of the protein are of great importance in this respect, but later on it was proved with rats and chickens that even when absolutely the same amounts of the different amino-acids are included in the diet, mixtures with animal protein yield better results. It followed that there must be another reason why animal proteins differ from vegetable proteins.

During 1942, workers at Cornell University put forward the suggestion that protein foods of animal origin contain a growth factor (or factors) to which the name of *animal protein factor* was given. Investigations to elucidate the nature of these factors were intensively prosecuted after that time, and it became probable that the animal protein factors themselves were not proteins, but belonged to the complex of the B-vitamins. It became evident that vitamin B_{12} is an important part of the animal protein factor. Trials on a large scale to test the value of vitamin B_{12} in animal feeding, followed as a consequence of these findings.

Several forms of vitamin B_{12} are known to exist now, namely B_{12}, B_{12a}, B_{12b}, B_{12c} and B_{12m}. Nowadays the basic complex of vitamin B_{12} is often called cobalamin. In this system the original vitamin B_{12} is named cyano-cobalamin and vitamin B_{12a} hydroxy-cobalamin (Kaczka, Wolf, Kuehl & Folkers, 1950).

Recently, Lewis, Tappan & Elvehjem (1952) have found a new modification of vitamin B_{12}, not identical with one of the forms mentioned above. This compound has growth-promoting properties for *Lactobacillus leichmannii* and for chicks, comparable to that of crystalline vitamin B_{12}. In rat tests, however, it proved completely inactive. This indicates that there may exist differences among the animal species in respect of the type of vitamin B_{12} they need. If this is correct, a careful examination of the testing methods, suitable for the different purposes for which these vitamins are intended, will become highly important.

Vitamin B_{12} can be obtained by fermentation under fixed conditions of protein-rich products, such as, for instance, soy-bean oil meal. Other sources of vitamin B_{12} are condensed fish solubles, condensed whey and dried whey. These are waste products of animal origin with low protein value.

By-products originating from the manufacturing of antibiotics also cause an increase of growth when added to the feed mixtures for pigs. The effects of these supplements are, however, more favourable than is consistent with the amount of vitamin B_{12} and should be ascribed to the presence of the antibiotic principle. This makes it clear that there is at present a sharp difference between the preparations obtained by bacterial fermentation and the preparations obtained by the manufacturing of antibiotics. The latter processes are in general based on the growth of moulds in a suitable substrate.

In the United States a large number of publications announce positive results from the use of vitamin B_{12} supplements for swine. It must be noted, however, that often the growth improvement is not very important (Vohs, Maddock, Catron & Culbertson, 1951; Bowland, Beacom & McElroy, 1951; Briggs & Beeson, 1951).

FEEDING TRIALS

In the Netherlands experiments have also been carried out to estimate the effect of the use of vitamin B_{12} in the rations of pigs. The difference between our experiments and those in the United States is in the first place the composition of the feed mixtures. In the United States the normal ration for fattening pigs consists only of soy-bean oil meal and maize meal, supplemented with vitamins, minerals and in some cases alfalfa meal. In Holland, on the other hand, the rations for swine are composed of far more ingredients.

In 1950 and 1951 a number of experiments were carried out. The results of the 1950 trials have already been published (Frens & Ubbels, 1951). The experiments were conducted on several farms and institutes, but were always set up along the same lines and with the same feed composition.

In all trials two control groups were used: one positive and one negative. The positive control groups received a ration with plenty of animal protein, chiefly in the form of fish meal or Norwegian herring-meal. The feed mixtures for the negative control groups contained less animal and more vegetable protein. For the pigs in these groups the herring-meal was entirely left out and only a small quantity of tankage or fish meal remained as source of animal protein. It is evident that both rations were comparable in respect of the amount of crude protein, starch equivalent and Ca/P ratio. The percentages of the different amino-acids in both rations were equalized so far as this was possible with regular feeding stuffs.

The composition of both rations is given in Table 1.

Table 1. *Composition of the feed mixtures*

	Negative control, 1950	Positive control, 1950	Negative control, 1951	Positive control, 1951
Ground barley	39	42	43	46
Ground maize	35	35	12	12
Wheat bran	12	12	10	10
Wheat middlings	—	—	9	9
Ground oats	—	—	13	14
Fish meal	—	—	2	8
Tankage	2	2	—	—
Norwegian herring-meal	—	8	—	—
Soybean oil meal	5	—	4	—
Sunflower seed oil meal	5	—	5	—
Mineral mixture and vitamin A–D_3 supplement	2	1	2	1
	100	100	100	100

Besides the two control groups, in every trial one or more test groups received the same ration as the negative control groups with the addition of one of the tested products. The following products were tried out in these experiments: (1) several commercial vitamin B_{12} preparations; (2) different kinds of condensed fish solubles; (3) condensed whale solubles; (4) dried and condensed whey.

The amounts of these products to be used was discussed with the producers.

Young pigs immediately after weaning have the greatest requirements for animal protein. Therefore we started the experiments with young pigs (average initial weight ± 20 kg.). The average final weight per group was about 60 kg. The test animals in all the trials were kept indoors. In all, 312 animals were used in the experiments.

RESULTS

In all the experiments the difference between the positive and the negative control groups turned out in favour of the first-mentioned. Though the difference was not always significant ($P < 0{\cdot}05$), we are confident that the results provide further confirmation of the contention that for pigs below 50 kg., part of the necessary protein should be of animal origin. When the results of both control groups differed markedly, the groups with the tested products in their feed showed a somewhat better growth than the negative control groups, but these differences were never significant. The results with the test groups, however, were not equal to those of the positive control groups. In general, growth was better when the ration contained plenty of animal protein.

The differences among the test groups were in all cases very small. No greater value could be ascribed to vitamin B_{12} supplements than to the other products.

The figures for feed efficiency showed the same tendency as those for the average gain. The positive control groups always gave the best results in this respect. Table 2 gives the figures from one of the experiments, representative of the whole series. This trial lasted 11 weeks and was carried out with groups counting eight animals.

Table 2. *Results of one of the trials*

	Negative control (1)	Positive control (2)	Negative control +0·1% vitamin B_{12} supplement (3)	Negative control +5% whale solubles (4)
Initial weight (kg.)	18·6	18·6	18·6	18·6
Final weight (kg.)	46·2	62·0	52·4	52·3
Mean daily growth (g.)	360	564	440	438
Standard deviation (g.)	±71	±29	±42	±22
Total feed consumed	825	1034	944	944
kg. feed/kg. growth	3·72	2·98	3·48	3·50
kg. starch equivalent/kg. growth	2·42	1·97	2·27	2·19

In some cases there was only little difference in gain between the positive and negative control groups. Naturally in these cases no effect of the tested products was observed. We suppose that the reason of the satisfactory growth of the negative control groups in these cases is related to the antecedents of the test animals. It is known that swine in their tissues can store a reserve of vitamin B_{12} (Wilder, 1951). Piglets of a sow which has received a ration with a sufficient amount of animal protein during pregnancy and lactation can have formed such a reserve. Piglets, on the other hand, farrowed by a sow which was fed a less balanced ration, will pro-

bably respond after weaning more clearly to a certain amount of animal protein in their feed mixtures.

If significant differences between the positive and the negative control groups appeared, the use of the tested products gave only a slight increase of the rate of growth. We have therefore concluded, that for pigs, a ration poor in animal protein cannot be made equal to a ration containing a normal content of animal protein by the addition of vitamin B_{12} supplements. Vitamin B_{12}, however, is of importance as a component of the so-called *animal protein factor*. Besides this, there must also be present some as yet unknown factors which give animal protein its special value. It is therefore not advisable to replace the animal protein in Dutch food mixtures, entirely or partly, by vegetable protein with addition of vitamin B_{12} supplements or other products which contain this vitamin.

SUMMARY

After it had become evident that vitamin B_{12} can be considered as a part of the so-called 'Animal Protein Factor', several experiments with young fattening pigs have been carried out in the Netherlands. The purpose of these experiments was to gain experience as to the value of products rich in vitamin B_{12} for pig feeding. In these trials 312 animals were used.

The results can be summarized as follows:

1. Substitution to a large extent of animal protein by vegetable protein in rations containing the same total percentage of protein resulted in a retardation of growth.

2. Addition of vitamin B_{12} supplements to feed mixtures low in animal protein in this series of experiments generally gave better results than could be obtained without this addition. In the majority of cases, however, the results did not equal those of the groups which received rations containing the usual amount of animal protein.

3. Vitamin B_{12} can be considered as a component of the animal protein factor, but besides this there probably exist other factors which contribute to the special value of animal protein for young pigs.

4. In circumstances such as prevail in the Netherlands, little benefit can be derived for pig fattening from the use of products rich in vitamin B_{12}.

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CYTOLOGICAL REGULATION OF VITAMIN-B_{12} ON NUCLEAR AND CE DIVISIONS

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Introduction

Hutner and co-workers (1957) have shown that *Euglena* and other flagellated algae need vitamin-B_{12} as growth factor. The requirement for B_{12} has to be raised 300x (times) when *Ochromonas malhamensis* is cultured at higher harvest temperature above 34°C, but it seems that very little attention has been given towards the regulation of B_{12} on the dynamic cellular cytology at the upper as well as the lower limits of harvest temperatures. In another set of experiments, Hutner *et al.* (1958) observed that Chlorophyceae needs B_{12} as stress factor only at upper or elevated harvest temperature. Two genetically different strains of algae with strong nutritional tendency for autotropic life, selected for the present experiments, have shown the specialized mode: "auxoautotropic" algal life under the regulation of B_{12} at both higher and lower limits of temperature. Attempts have also been made to represent the cytological picture of the cell and the nucleus for better understanding of the B_{12}-effect.

Experimental data

Pseudochlorella, a material isolated from a lichen, *Stereocaulon alpinum* Laur, prefers to grow always below 30°C under 20 Kilolux light intensity. The cytological regulation of B_{12} (50 mμg/ml) in the defined mineral nutrient solution at that stage offers the low-temperature strain, *Pseudochlorella*, to lead an auxo-autotropic mode of life. Feulgen-nuclear reactions reveal the vitamin action represented by microphotography on red-sensitive film as shown in fig. 1. The periodic light/dark phases for the strain bring about also the similar experimental result showing the need of darkness on cellular multiplication, without B_{12} requirement.

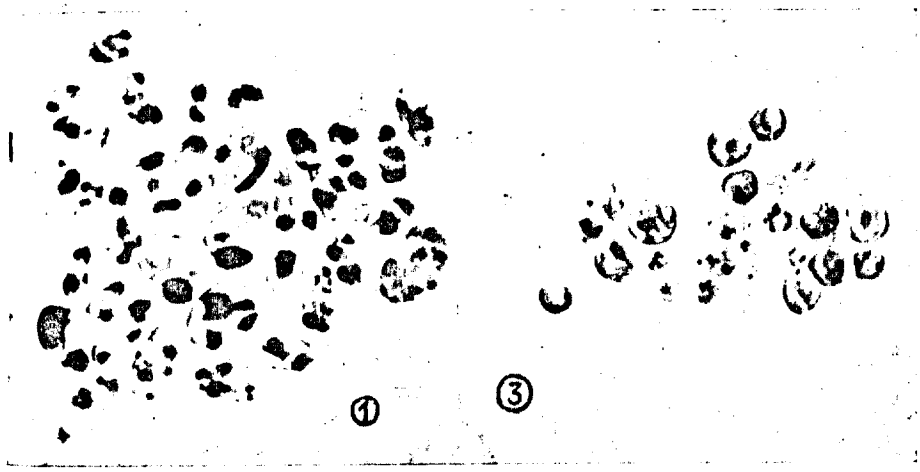

Fig. 1. Influence of B_{12} on cell and nuclear divisions. *Pseudochlorella* at 30°/20 Kilolux.
Fig. 3. Influence of B_{12} on cell and nuclear divisions. *Chlorella* 211/8K at 15°/20 Kilolux.

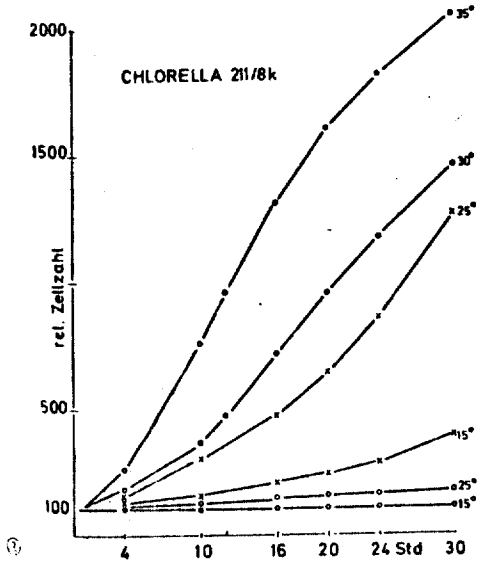

Fig. 2. Regulation of Vitamin B_{12} (x——x) on cell-multiplication towards experimental tempera tures at: 15, 25, 30 and 35°C. Control without B_{12} (o——o). Comparison with optimum temperature at 30°. (rel. Zellzahl, i.e. relative cell-count, $100 = 1{\cdot}5 \times 10^6$ cells and Std., i.e. hours), *Chlorella* 211/8K.

The high-temperature strain *Chlorella* 211/8K shows its optimum growth at 30°C under 20 Kilolux. But it has been reported to go up to 39°C (Sorokin, 1959). At 15 and 25°C it cannot lead an autotropic life. Fig. 2 shows the pattern of cellular multiplications with and without B_{12} auxiliary action. The effect of B_{12} at 25°C under same illumination is clearly represented reaching almost to the optimum at 30°C where the cells are well autotrophs. The stay at 15°C induces a slight increase in cell population but more towards death under 20 Kilolux causing fading-out of chlorophyll pigments (eventually also for selection of stable mutants by the few survivals). The cells are able to normalise their life activities under the cytoplasmic control of B_{12} on both nuclear

and cell divisions, (fig. 3). The figure 4 represents the schematic demonstration on the control mechanism. Within 30-50 hours B_{12} regulates the so-called "giant cells" formation at 15°C and they are totally checked at 25°C where the effect is quite clear on the active cell division.

Conclusions

It has also been observed that B_{12} as promoter substance, influen the photosynthetic 0_2 production (Das, 1968) for the autotropic alga. This vitamin here as a c for both th strains helpin in normal ce division from the tory and phy cal effects of nuous illumination at *two* limits (upper and lower) of temperature. The action has also been shown comparable to dark phase in periodic light/dark experiments (in *Chlorella*, where it seems that in addition to photosynthesis, other factors are involved. With regard to these, the cells presumably show insufficient DNA-production to the dividing level for the cells. Actual analysis shows that the cellular level is controlled by a block. The B_{12} effect removes it by taking part as co-factor in the cellular nucleic acids level,—under such physiological attainments of the cells where the building constituents of DNA are in attacking stage. The replacement of B_{12} with the major nucleosides proves the validity of the inference, on which data are being published elsewhere.

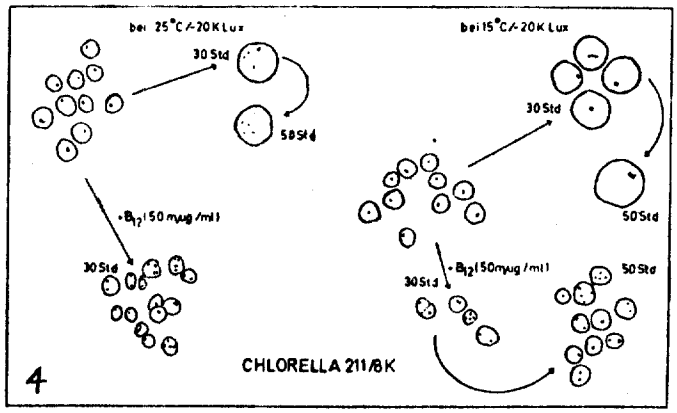

Fig. 4. Cytoplasmic regulation of Vitamin-B_{12} on nuclear and cell-divisions. Chlorella 211/8K at 25 and 15°C with 20 Kilolux. Cells raised from light/dark 12 : 12 hours at 25°C. (Std. = Hours).

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The Absorption of Oral Vitamin B_{12} in an Aged Population[1,2]

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Chow, Davis, and Lawton (1962) have reported studies in which different dosages of vitamin B_{12} in the free form or in combination with resins were administered daily to young and old *Ss* selected from relatively homogeneous populations. They observed that vitamin B_{12} adsorbed to resin was more efficiently absorbed than free vitamin B_{12} by both groups, one of which had an average age of 70 years. Since Mollin and Ross (1952) reported that a group of elderly patients had a significantly lower mean serum vitamin B_{12} level than the healthy hospital staff under 40 years of age, other investigators (Boger, Wright, Strickland, Gylfe, & Ciminera, 1955; Chow, Wood, Horonick, & Okuda, 1956; Gaffney, Horonick, Okuda, Meier, Chow, & Shock, 1957) have reported decreases in serum vitamin B_{12} levels with advancing age. In a study of 62 men, average age 86 years, Davis, Lawton, and Barrows (1963) reported not only a significant lowering of this vitamin with age but also that over 10% of these *Ss* had serum vitamin B_{12} levels which approached the pernicious anemia range ($\sim 50 \mu\mu g$/ml.). These depressed serum vitimin B_{12} levels in this aged group stimulated our interest in determining whether the absorptive capacity of the aged was impaired and whether any significant increments in vitamin B_{12} absorption would be reflected as an improved sense of well being, weight gains, and improved appetite.

Materials and Methods

Design of study.—In this study, random distribution of *Ss* was attempted by placing the individual names of the 60 *Ss* on separate index cards, which were mixed thoroughly. The names were then listed on a tabulation sheet according to the order obtained after shuffling. They were also assigned randomly by the same method into the four test groups, which were to be given, viz., aqueous B_{12}, Pfizer 231-1, Pfizer 231-2, or vitamin B_{12}-Mg. Trisilicate. All participants of the study received the equivalent of 20 μg of vitamin B_{12} for 12 weeks.

Subjects.—The population was composed of 45 residents of a VA Domiciliary and 15 hospital patients who were transferred from the VA Domiciliary for physical ailments (orthopedic), which would not have an effect on their serum vitamin B_{12} levels or their vitamin B_{12} absorption. All *Ss* were free of overt acute diseases and were ambulatory. The average age of these *Ss* was 86 (range 81-99) years. None showed clinical or laboratory evidence of significant liver or renal disease. Their diets included generous amounts of meat, milk, eggs, and other nutrients. The daily caloric intake for both groups was estimated at 2700 calories/day. Each *S* was examined every two weeks by a VA Domiciliary physician. The body weight and nutritional history of the previous two weeks' clinical status were reviewed during this biweekly checkup.

Microbiological assay of vitamin B_{12}.—The technique for microbiological assay for vitamin B_{12} activity of the serum samples was that of Skeggs, Happle, Valendik, Huff, and Wright (1950). The accuracy and reproducibility of the microbiological analysis as well as the stability of frozen samples have been reported elsewhere (Chow et al., 1962).

Fasting blood samples were drawn from *Ss* before vitamin administration (basal), 6 and 12 weeks after vitamin administration, and finally, 3 and 6 months after termination of

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administration of vitamin B_{12} preparations. Serum was separated immediately and shipped or delivered in a frozen state to be stored in a laboratory freezer at the Department of Biochemistry, John Hopkins University, School of Hygiene and Public Health, until it could be analyzed microbiologically. Previous data have demonstrated that the microbial activity of vitamin B_{12} in serum is not altered under these conditions (Chow et al., 1962).

Vitamin B_{12} preparations.— A: The aqueous vitamin B_{12} solution was prepared from crystalline vitamin B_{12} Merck and distilled water. B: Pfizer 231-1 consisted of crystalline vitamin B_{12} (1%) adsorbed on an ion exchange resin (Rohm & Haas IRC 50, 99%). C: Pfizer 231-2 consisted of crystalline vitamin B_{12} (1%) physically blended with mannitol (99%). The two products described above were provided by Charles Pfizer & Co., Inc., New York, New York. D: Warner Lambert vitamin B_{12}-magnesium trisilicate preparation consisted of 25 μg. crystalline cyanocobalamin combined with 45 mg. magnesium trisilicate (preparation described in United States Patent 3,060,095). This product was provided by Warner Lambert Research Institute, Morris Plains, New Jersey.

Results

The 60 *Ss*, average age 86, who had been divided into groups as described, were diminished by 3 deaths, 3 transfers, and 10 dropouts, so that only 44 completed the study. Table 1 summarizes the data obtained from the oral administration of the four preparations of vitamin B_{12}. As indicated in Table 1, from an initial basal level of approximately 150 $\mu\mu$g/ml., all groups showed a serum vitamin B_{12} increase of approximately 100 $\mu\mu$g/ml. after 6 weeks of treatment and approximately 150 $\mu\mu$g/ml. after the 12-week period. The group which received the Pfizer 231-2 preparation of B_{12} showed the largest increment (200 $\mu\mu$g/ml.) over the test period. The aqueous vitamin B_{12} group showed the smallest serum rise (less than 140). The serum vitamin B_{12} levels of the four test groups were higher after 12 weeks of treatment than after 6 weeks of treatment. In all groups, the basal levels were reached no later than 12 weeks after cessation of vitamin B_{12} treatment.

Table 1. Effect of Daily Oral Administration of 20 MCG of Vitamin B_{12} or Vitamin B_{12} Complexes on Mean Serum Levels of Vitamin B_{12}.

	Serum Vitamin B_{12} Levels $\mu\mu$g/ml. ± S.E.mn				
		Test Period (Weeks)		Post-Treatment (Weeks)	
Group	Basal	6	12	12	24
Aqueous B_{12} (A)	156 (15)[a]	260±30 (14)	291±37 (12)	148 (11)	140
Pfizer 231-1 (B)	150 (15)	285±62 (13)	312±31 (11)	160 (10)	150
Pfizer 231-2 (C)	161 (15)	281±51 (15)	365±39 (12)	158 (12)	171
B_{12}-MG (D) Trisilicate	145 (15)	265±28 (15)	342±52 (12)	178 (11)	168

[a] ( ) Number in group.

The mean serum vitamin B_{12} levels $\pm S.E_{Mn}$ of the total population during the various test periods are presented in Figure 1. The mean serum level increased from the basal level of approximately 150 $\mu\mu$g/ml. to slightly over 300 $\mu\mu$g/ml. at the end of the 12th week of administration of the vitamin B_{12} preparations. It is interesting to note that 12 weeks after institution of vitamin administration the level was twice that of the basal (150 vs. 300 $\mu\mu$g/ml.). Mean basal level was attained 12 weeks after termination of administration of the vitamin B_{12} preparations.

Figure 2 illustrates data from two representative *Ss* with significantly different initial basal levels (50 vs. 205 $\mu\mu$g/ml.). The significant increase in the serum vitamin B_{12} levels of both *Ss* at the end of the 12-week administration

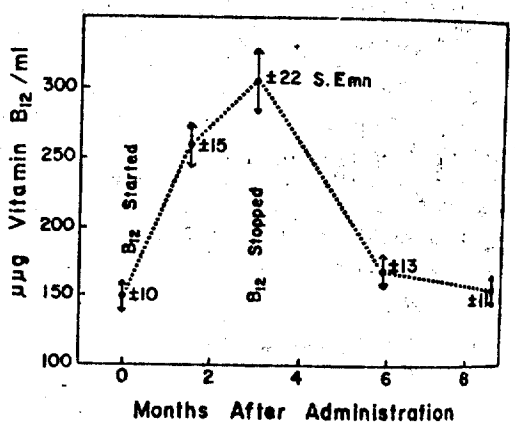

Fig. 1. Mean serum vitamin B_{12} levels of 40 Domicile members, average age 87, after oral administration of 20 μg vitamin B_{12}. Vertical lines represent standard error for each mean value. Each point represents the average of microbiological assays of 40 serum vitamin B_{12} determinations of Domicile members who received 20 μg of the four vitamin B_{12} preparations described earlier, during 12-week test period.

period is shown in Figure 2. After termination of vitamin B_{12} administration, the serum vitamin B_{12} level of both *Ss* leveled off to about the same concentrations. In other *Ss* with low basal levels (~75 $\mu\mu$/ml.), no consistent patterns of serum vitamin B_{12} levels after the test periods were observed. The same observation was noted for those with higher levels (> 175 $\mu\mu$g/ml.). The serum vitamin B_{12} increments after oral administration of vitamin B_{12} in both groups studied were not significantly different.

Preparations administered to group B and D (Pfizer 231-1 and vitamin B_{12}-Mg. trisilicate) produced increments intermediate to groups A and C. These results compare favorably to those reported from a group of young women (Chow et al., 1962) who showed an increment of 180 $\mu\mu$g/ml. when administered 25 $\mu\mu$g of vitamin B_{12}-resin daily for two months.

Discussion

Our data indicate no impairment in vitamin B_{12} absorption in this "aged" population. The increment in serum vitamin B_{12} following the 12-week period of treatment was very similar to that of a young group (Chow et al., 1962). Gaffney, Watkin, and Chow (1959) reported that there were no demonstrable age differences in urinary excretion of orally administered $Co^{60}B_{12}$ for any of the doses used in their study, which included lower and higher oral doses than those of this report. If the urinary excretion of orally administered vitamin B_{12} is useful to estimate the absorption of this vitamin

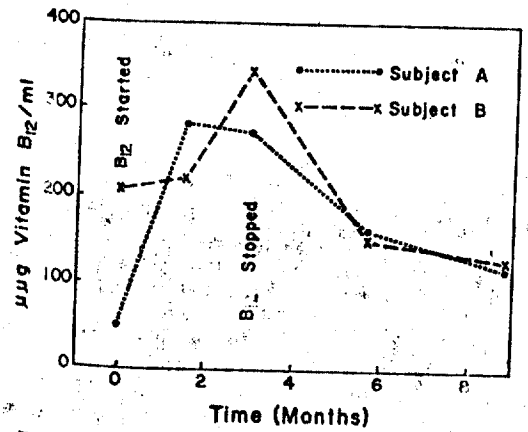

Fig. 2. Vitamin B_{12} serum levels of two *Ss* (low and high basal serum vitamin B_{12} level) who received 20 μg vitamin B_{12}-resin complex during the 12-week test period. *S* A-basal level 205 $\mu\mu$g/ml. *S* B-basal level ~ 50 $\mu\mu$/ml.

(Gaffney et al., 1959; Schilling, 1953), then their data suggest that both groups have a similar absorptive capacity for vitamin B_{12}.

The increments of serum vitamin B_{12} levels of the old *Ss* cannot be explained by a regression of renal function with age. If so, urinary excretion after a test dose of Co^{60} vitamin B_{12} would be suppressed in the aged. Gaffney et al. (1959), on the contrary, found that after an oral dose of Co^{60} vitamin B_{12}, the urinary excretion of their elderly *Ss* contained as much of the administered radioactivity as did their young group. It is also of interest to note that Glass, Goldbloom, Boyd, Loughton, Rosen, and Rich (1956) did not observe any significant difference in the amount of radioactive vitamin B_{12} retained by the liver of the young and old *Ss* after oral administration of vitamin B_{12}.

Ranke, Tauber, Horonick, Ranke, Goodhart, and Chow (1960) reported the existence of a mild state of vitamin B_6 deficiency among the aged as demonstrated by lowered serum transaminase (SGOT) and increased excretion of xanthuremic acid after a tryptophane load test. In view of the report of Hsu and Chow (1957), which indicated that vitamin B_6 deficiency in the rat will reduce the absorption of vitamin B_{12}, it is tempting to speculate that this may be a factor in limiting the absorption of vitamin B_{12} when ingested by the aged *S* in the low concentrations present in their diet.

Absorption of vitamin B_{12}, when ingested in larger amounts (20 μg/day) may be proportionately greater in the old *S* than when administered in smaller amounts due to a mechanism relatively independent of the amount of intrinsic factor available (Gaffney et al., 1959). The oral administration of 20 mg. on a daily basis for the 12-week test then could account for the relatively good absorption of the aged *Ss*. Chow et al. (1962) reported no demonstrable increase in serum vitamin B_{12} level of 19 aged *Ss* who received 2 μg free vitamin B_{12}, although 2 μg of vitamin B_{12}-resin resulted in an 100 $\mu\mu$g/ml. increase.

It is possible that the low basal levels observed in a large number of our aged population is partially the result of long-term decreased intake of vitamin B_{12}, because of dietary selection of the *Ss* themselves (Gaffney et al., 1957). In our biweekly ten-minute interviews with our *Ss* with both low and high basal serum vitamin B_{12} levels, we did not observe any noteworthy

differences in dietary habits between the two groups.

The significant absorption of vitamin B_{12} (either in free form or combined with resin) by our aged population can be explained, in part, by the efficiency of such a mechanism which does not differ from a younger population (Gaffney et al., 1959) when this vitamin is ingested in larger amounts.

It is interesting that hemoglobin and hematocrit determinations performed earlier on the *Ss* divided into those with higher ($> 150\ \mu\mu g$/ml.) and those with lower ($\sim 80\ \mu\mu$/ml.), basal serum vitamin B_{12} levels were not significantly different. The biweekly observations and interviews of the *Ss* during this study by the Domiciliary physician indicated that there were no significant objective changes in weight, appetite, or sense of well-being. A large percentage of the participants volunteered the information that there was an improvement in their appetites. An equally large number noted no change and a few complained of lessening of appetite during the test periods.

Summary

The absorption of free vitamin B_{12} or vitamin B_{12} adsorbed on resin or on magnesium trisilicate was measured in 44 *Ss* aged 81-89 years (average 86 years). The results of microbiological assays of serum samples for total vitamin B_{12} content obtained prior to, during, and after the oral administration of 20 μg of vitamin B_{12} in four different forms for three to six months, indicated that an aged population absorbed considerable quantities of this vitamin independent of their basal levels. The oral administration of vitamin B_{12}-resin in complexes resulted in larger increases in serum vitamin B_{12} levels than that of free vitamin B_{12}. Serum vitamin B_{12} levels of all groups returned nearly to pretest basal levels within three months after termination of vitamin B_{12} administration. There were no observable differences in weight, appetite, and sense of well-being during test period.

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Procarcinogenic Effect of Vitamin B_{12} on p-Dimethylaminoazobenzene-Fed Rats.* (18067)

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Diet is known to influence the carcinogenicity of p-dimethylaminoazobenzene (DAB). Riboflavin has been shown to exert a protective effect(1) while it has been reported that biotin is procarcinogenic(2). The experiments to be reported in this paper indicate that vitamin B_{12} exerts a marked procarcinogenic effect on DAB-fed rats.

Experimental. Weanling female Sprague-Dawley rats were fed a purified diet consisting of the following: isolated soybean protein,† 18 g; sucrose, 67.4 g; hydrogenated vegetable oil, 8 g; cod liver oil, 2 g; salt mixture(3), 4 g; choline chloride, 0.1 g; inositol, 10 mg; thiamine chloride, 1.5 mg; riboflavin, 0.5 mg; nicotinamide, 2 mg; calcium pantothenate, 1.0 mg; pyridoxine hydrochloride, 0.5 mg; vitamin K, 0.025 mg; biotin, 0.005 mg; folic acid, 0.5 mg; and p-dimethylaminoazobenzene, 70 mg. This diet contains approximately 0.27% methionine. One group of rats received this diet unsupplemented, one group received this diet plus 5 micrograms of vitamin B_{12} (Rubramin) per 100 g, a third group received this diet plus 0.6% DL-methionine, and a fourth group received the

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TABLE I.
Influence of Vitamin B_{12} and Methionine on Incidence of Hepatomas in p-Dimethylaminoazobenzene-Fed Rats.

Supplement to diet	Avg daily food intake, g	Avg daily wt gain, g	Incidence of hepatomas, %	Liver wt*— % of body wt
None	6.4	0.1	17	9.3
Vitamin B_{12}	7.0	0.2	78	15.7
Methionine	8.1	1.2	11	7.0
Methionine + vit. B_{12}	10.5	1.2	33	11.0

* Liver weights include tumors, where present.

diet supplemented with both vitamin B_{12} and methionine. The rats were fed these diets for 170 days and were then killed and autopsied. The incidence of gross hepatomas was recorded. There were 9 rats in each group. Three rats in the unsupplemented group died without hepatomas during the experimental period. Two rats receiving vitamin B_{12}, one rat receiving methionine, and one rat receiving vitamin B_{12} plus methionine died with hepatomas during the experimental period. The incidence of hepatomas is based on those rats either surviving the 170-day period or dying with hepatomas.

Results and discussion. The pertinent data are presented in Table I. The addition of vitamin B_{12} to the DAB-containing, methionine-deficient diet increased the incidence of hepatomas from 17% to 78%. The addition of vitamin B_{12} to the methionine-containing diet increased the incidence of hepatomas only from 11% to 33%; thus methionine appeared to afford some protection.

The procarcinogenic effect of vitamin B_{12} may indicate that this vitamin is required for tumor growth or it may be a reflection of an influence of vitamin B_{12} on metabolic transformations of the carcinogen p-dimethylaminoazobenzene. In the former event these results might have general application to the entire field of cancer. Experiments are in progress to elucidate the mechanism of the procarcinogenic effect of vitamin B_{12}.

It would be unfortunate if the conclusion were drawn from these experiments that vitamin B_{12} itself is carcinogenic. There is no evidence that such is the case. On the contrary, a control group of rats receiving the soybean protein diet with vitamin B_{12}, but without p-dimethylaminoazobenzene, showed no hepatic tumors.

Summary. Vitamin B_{12} has been found to enhance markedly the carcinogenic effect of p-dimethylaminoazobenzene in rats receiving a methionine-deficient diet. However, a control group of rats receiving this diet without p-dimethylaminoazobenzene showed no hepatic tumors.

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The role of propionic acid as a precursor of methylmalonic acid in normal and vitamin B_{12}-deficient man

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In order to clarify the role of propionic acid as a precursor of methylmalonic acid (MAA) in the intact human, measurements of the incorporation of intravenously administrated tracer amounts of 2 ^{14}C-propionate into urinary ^{14}C-MMA, miscible MMA pool size, turnover, and urinary excretion were performed in vitamin B_{12}-deficient patients and suitable controls. While miscible MMA pool size is markedly increased in vitamin B_{12} deficiency (10 to 20 times), the fractional turnover rate is similar in controls and vitamin B_{12} deficiency. The daily absolute turnover and urinary excretion is proportional to MMA pool size. MMA specific activity following intravenous ^{14}C-propionate, however, was similar in all groups despite marked differences in pool size. These findings demonstrate that propionate is a precursor to MMA in normal and B_{12}-deficient man and, furthermore, suggest that the fraction of propionate going to methylmalonyl CoA and the fractional participation of propionate in relation to other precursors of methylmalonyl CoA is unchanged in vitamin B_{12} deficiency.

The increased methylmalonic acid (MMA) excretion of vitamin B_{12} deficiency[1-5] and certain congenital disorders[6-8] is the result of diminished isomerization of methylmalonyl-CoA to succinyl CoA by the vitamin B_{12}-dependent methylmalonyl-CoA isomerase system.[9, 10]

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Although propionic acid is a major precursor of methylmalonyl-CoA in ruminants,[11] its exact relationship to MMA in the intact man remains obscure.

The present study includes measurements of the incorporation of intravenously administered tracer amounts of 2^{14}C-propionate into urinary ^{14}C-MMA, MMA pool size, turnover, and urinary excretion so as to determine the role of propionic as a precursor of MMA in man.

Methods and materials

Subjects consisted of a control group and a vitamin B_{12}-deficient group. The control group included 2 normal subjects and 2 patients with partially treated folic acid deficiency. The vitamin B_{12}-deficient group consisted of 4 patients with pernicious anemia. Also studied were 2 vitamin B_{12}-deficient patients 25 days and 10 months after the onset of vitamin B_{12} therapy. All subjects with vitamin B_{12}-deficiency had characteristic megaloblastic bone marrow changes. The vitamin B_{12} and folic acid status of the subjects was determined by serum vitamin B_{12}[12] and folate levels,[13] ^{57}Co-vitamin B_{12} absorption studies,[14, 15] daily urinary MMA excretion,[16] $^{14}CO_2$ breath excretion patterns after i. v. administration of 2(ring) ^{14}C-histidine and 2^{14}C propionate[17, 18] and when possible, reticulocyte response to physiological therapy.[19, 20]

Fasting subjects received an intravenous injection of 10 to 15 μCi of 2^{14}C-propionate (specific activity 8.15 mCi/mM.—radiochemical purity $>$ 99 per cent)* and/or 25 μCi of 2^{14}C-MMA (specific activity 1.9 mCi/mM., radiochemical purity $>$ 98 per cent).† One vitamin B_{12}-deficient patient also received a 5.0 Gm. oral dose of sodium propionate 30 minutes prior to i. v. injection of 2^{14}C-propionate. When multiple studies were performed on a single subject, a minimum of one week was allowed to elapse between studies. Fractional urine collections were obtained in bottles containing thymol for 48 hours after injection and were frozen at 20° C. until time of analysis.

Urinary MMA and ^{14}C-MMA specific activity were quantitated by methods previously described.[16] These methods include ether extraction, anion exchange chromatography, gel filtration, and spectrophotometric quantitation of the diazotized isolated MMA. ^{14}C-MMA specific activity was determined by further steps including thin-layer silica gel chromatography and liquid scintillation counting.

Urinary ^{14}C-MMA specific activity was expressed as a percentage of the radioactivity administered as either ^{14}C-propionate or ^{14}C-MMA per milligram of urinary MMA. MMA pool size and daily turnover was determined by in vivo isotope dilution techniques similar to that applied to the measurement of uric acid pool and turnover.[21] This technique involves serial measurement of urinary ^{14}C-MMA specific activity after intravenous injection of ^{14}C-MMA. The miscible MMA pool size is determined by relating the zero time extrapolated specific activity to the amount of ^{14}C-MMA injected. Turnover rate of the miscible MMA pool is determined from the slope of the serial ^{14}C-MMA specific activity determinations. The daily turnover of MMA is the product of the miscible MMA pool and its turnover rate.

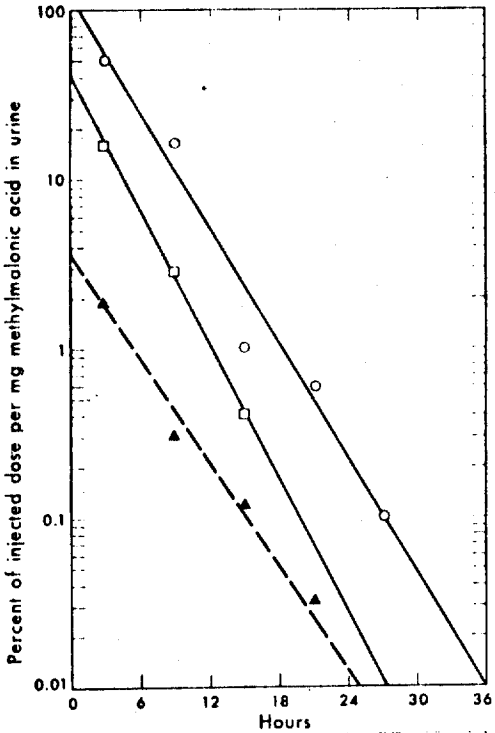
Results

While our primary objective was to determine the role of propionate as a precursor of MMA by quantitating ^{14}C-propionate incorporation into urinary MMA, knowledge of the MMA pool size and turnover aids in the interpretation of such data. Serial ^{14}C-MMA specific activity determinations on 6 hour fractional urine collections after i. v. injection of ^{14}C-MMA into 2 normal subjects and one patient with vitamin B_{12}-deficient megaloblastic anemia is shown in Fig. 1. The specific activity, expressed as a percentage of the injected dose of ^{14}C per

*Nuclear-Chicago Corp., Des Plaines, Ill.
†New England Nuclear Corporation, Boston, Mass.

milligram of MMA, is plotted against the mean time of each urine collection interval. During the 48 hour period of measurements, the ^{14}C-MMA specific activity is noted to decrease at a single exponential rate with half-times of 2.7 and 2.3 hours in the normal subjects and 3.0 hours in the vitamin B_{12}-deficient patient. The calculated miscible MMA pool, its fractional turnover rate, and quantitative daily turnover as well as the 24 hour urinary MMA excretion of these same subjects are also listed in Fig. 1. Miscible MMA pool size (27.8 mg.) in this vitamin B_{12}-deficient patient is approximately 10 to 20 times greater than that of the normal subjects (1.0 and 2.4 mg.).

It is of interest to note that the fractional turnover rate is similar in both normal controls and vitamin B_{12} deficiency, and consequently the daily turnover



	Pool size (mg)	Turnover rate (fract/day)	Turnover (mg/day)	Urinary excretion (mg/day)
○ CONTROLS	1.0	6.1	6.1	3.5
□	2.4	7.4	17.7	3.2
▲ B_{12} DEFICIENT	27.8	5.5	152.6	35.9

Fig. 1. Serial urinary ^{14}C-MMA specific activity measurements after i. v. injection of ^{14}C-MMA into one vitamin B_{12}-deficient patient, ▲; and two normal controls, ○ and □. The calculated MMA miscible pool size, turnover rate, absolute turnover, and MMA urinary excretion are listed for these subjects.

Table I. Urinary ^{14}C-methylmalonic acid after i. v. 2^{14}C-propionate

	Urinary methylmalonic acid (mg./48 hr.)	*Per cent injected dose in methylmalonic acid per 48 hr. (%)*	*Methylmalonic acid specific activity (% inj. dose/mg.)*
Normal controls (4)	10.3 (4.6-13.4)	0.05 (0.02-0.11)	6.5×10^{-3} ($1.5\text{-}11 \times 10^{-3}$)
Treated vit. B_{12} deficiency (1)	5.8	0.04	7.2×10^{-3}
Partially treated vit. B_{12} deficiency (1)	73.7	0.55	7.5×10^{-3}
Vit. B_{12} deficiency (4)	1,086 (54-3477)	2.51 (0.47-8.10)	6.4×10^{-3} ($1.0\text{-}14.0 \times 10^{-3}$)

Table II. Urinary ^{14}C-methylmalonic acid after i. v. 2^{14}C-propionate before and after propionate loading in a patient with vitamin B_{12}-deficient megaloblastic anemia

	Urinary methylmalonic acid (mg./48 hr.)	*Per cent injected dose in methylmalonic acid per 48 hr. (%)*	*Methylmalonic acid specific activity (% inj. dose/mg.)*
Normal diet	3,477	8.1	2.30×10^{-3}
Normal diet plus 5 Gm. of sodium propionate	5,809	17.4	2.84×10^{-3}

is proportional to the MMA pool size. Urinary excretion of MMA is also proportional to pool size but represents only 20 to 50 per cent of the total turnover.

Urinary MMA excretion and ^{14}C-MMA determinations after injection of trace amounts of 2^{14}C-propionate into 4 controls, fully treated and partially treated patients with vitamin B_{12} deficiency, and 4 patients with vitamin B_{12}-deficient megaloblastic anemia are shown in Table I. The fully treated patient had received 1000 μg of vitamin B_{12} i.m. monthly for 10 months. The partially treated patient received 1 μg of vitamin B_{12} i.m. per day for 25 days. The results are expressed in terms of 48 hour urine collections after administration of the ^{14}C-propionate, because it was found that essentially all of the excretion of radioactivity in the MMA fraction occurred within 48 hours. In all the subjects studied, incorporation of the 2-carbon atom of propionate into urinary MMA is noted to occur. While there is a marked increase in MMA pool size and turnover in the partially treated and vitamin-deficient patients, no significant difference in ^{14}C-MMA specific activity was noted between these patients and the controls. Therefore, the increased percentage excretion of the injected dose as ^{14}C-MMA in the partially treated and vitamin B_{12}-deficient patients was due to the increased excretion of MMA in these patients.

The effects of a 5.0 Gm. oral propionate load on urinary MMA excretion and ^{14}C-MMA determinations after injection of ^{14}C propionate in a vitamin B_{12}-deficient subject are shown in Table II. On a normal diet this subject excreted very large amounts of MMA. While the oral propionate load resulted in a marked increase in MMA excretion and MMA pool size, no significant change is noted in the ^{14}C-MMA specific activity. Thus doubling of the MMA pool size by propionate loading in the same subject did not change ^{14}C-MMA specific activity. This constancy of specific activity in a single subject is similar to that noted in the subjects with widely varying MMA pool size and urinary excretion (Table I).

Discussion

The variable degree of excessive methylmalonic aciduria noted in vitamin B_{12} deficiency suggests that there may be multiple dietary and endogenous precursors of urinary MMA in man. Thus the excessive degree of methylmalonic aciduria noted in a given patient may be not only dependent upon the degree of metabolic block but also upon the types and amounts of available precursors which may vary from patient to patient. Substances that have been considered as possible precursors of MMA in man include propionic acid and other odd-numbered fatty acids, isoleucine, valine, cholesterol, and possibly pyrimidine catabolites.[22-24]

Propionic acid, a product of rumen bacterial action via its activation to propionyl-CoA, is a major precursor of methylmalonyl CoA and a major source of energy in ruminants.[11] The quantitative relationship of propionic acid to MMA in man is unknown. While the oral intake of valine has a relatively predictable effect on urinary MMA output in patients with diminished methylmalonyl-CoA isomerase activity,[23] oral loading doses of sodium propionate have failed to uniformly and predictably increase urinary MMA excretion.[2, 23, 25] Although the oxidation of 2-^{14}C-propionate has been found to be abnormal in human vitamin B_{12} deficiency, the degree of oxidative abnormality did not correlate with the degree of methylmalonic aciduria. Furthermore, after adequate vitamin B_{12} therapy, the oxidative abnormality persisted long after the urinary MMA excretion returned to normal.[18]

The results of the present tracer studies (Tables I and II) and loading study indicate that propionate is a precursor to urinary MMA and therefore intracellular methylmalonyl-CoA in both normal and vitamin B_{12}-deficient human subjects. While the percentage of injected activity found in urinary MMA was relatively small, especially in the cases of the nonvitamin B_{12} subjects, the MMA pool size and turnover studies indicate that urinary excretion accounts for only approximately 20 to 50 per cent of the daily MMA turnover in normal subjects and approximately 20 per cent in vitamin B_{12} deficiency (Fig. 1). The metabolic fate of the remainder of the daily MMA turnover is unknown. Preliminary studies in this laboratory have shown that after injection of ^{14}C-MMA, 20 per cent of the dose is expired in the breath as $^{14}CO_2$.

The marked augmentation of the methylmalonic aciduria by oral propionate loading in the vitamin B_{12}-deficient patient in the present study is noteworthy as prior attempts to increase the methylmalonic aciduria in vitamin B_{12} deficiency

by such loading have resulted in inconstant and minimal increases in urinary MMA.[2, 23, 25] The fact that this patient had an extreme degree of methylmalonic aciduria prior to loading may in some way relate to the marked augmentation after loading. Diminished catabolism of propionate by normally operative metabolic pathways not dependent upon vitamin B_{12} could explain this occurrence.[22, 26]

Even though there is a marked increase in the MMA pool in vitamin B_{12} deficiency, there was no significant difference in urinary ^{14}C-MMA specific activity after injection of ^{14}C-propionate into controls and vitamin B_{12}-deficient subjects, or change in ^{14}C-MMA specific activity after increase in pool size by oral propionate loading. These findings indicate that the fraction of propionate pool going to methylmalonyl-CoA and the fractional participation of propionate in relation to other precursors of methylmalonyl-CoA is unchanged in vitamin B_{12} deficiency.

These results suggest that the methylmalonyl-CoA pool size may not be appreciably altered by vitamin B_{12} deficiency even though there is diminished isomerization of methylmalonyl-CoA to succinyl CoA. A compensating increase in the rate of deacylation of methylmalonyl-CoA to MMA would maintain the methylmalonyl-CoA pool at its normal size.

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STUDIES ON THE ROLE OF VITAMIN B_{12} FOR THE SYNTHESIS OF METHIONINE IN LIVER

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The methyl transfer from N^5-methyltetrahydrofolic acid (CH_3THF)† to homocysteine, in *Escherichia coli* extracts, is one of the known metabolic reactions with an *in vitro* cobamide requirement.[1,2,3] Other cofactors required for this terminal step of methionine biosynthesis are reduced flavin[4,5] and S-adenosylmethionine (AMe).[6] The chemical synthesis of 5,6-dimethylbenzimidazolylcobamide methyl (CH_3B_{12}) by Smith *et al.*[7] has made it possible to test this compound as a methyl donor during methionine biosynthesis. Guest and coworkers[8] demonstrated that CH_3B_{12} could replace the requirement for vitamin B_{12} and, of more significance, could transfer its methyl group to homocysteine to form methionine. These experimental findings support the concept that protein-bound CH_3 B_{12} is the active form of the vitamin involved in methionine biosynthesis in bacteria.

Other workers have also shown that, in animal tissues, the transfer of the methyl group from CH_3THF to homocysteine is the terminal step of methionine biosynthesis.[9] No *in vitro* cobamide requirement in animal tissues has as yet been reported[9,10] but S-adenosylmethionine has been shown to be required.[11] However, none of the reported enzyme systems has been extensively purified.

The necessity for more complete information about methionine biosynthesis in animal tissues becomes apparent following a review of the nutritional interrelationships between methionine, folic acid, and vitamin B_{12}. Methionine has been shown to exert a sparing effect on the vitamin B_{12} requirement in chickens[12,13,14] and also to decrease the formiminoglutamic acid excretion observed in vitamin B_{12} deficiency in rats and chickens.[15,16] Recently, the feeding of methionine or glycine has been reported to decrease the elevated formiminoglutamic acid excretion in patients with pernicious anemia or megaloblastic anemia of pregnancy.[17]

The present study concerns the *in vitro* biosynthesis of methionine from CH_3THF or CH_3 B_{12} with the object of clarifying the possible role of vitamin B_{12} in this reaction in animal tissues. The necessary cofactor requirements for methionine synthesis, the purification of the enzyme

* This work was done during the tenure of an Established Investigatorship of the American Heart Association.

† Abbreviations: CH_3THF, N^5-methyltetrahydrofolic acid; AMe, S-adenosylmethionine; CH_3B_{12}, 5,6-dimethylbenzimidazolylcobamide methyl; vitamin B_{12} 5,6-dimethylbenzimidazolylcobamide cyanide; B_{12} coenzyme, 5,6-dimethylbenzimidazolylcobamide 5′-deoxyadenosyl; B_{12r}, reduced vitamin B_{12} containing divalent cobalt; ATP, adenosine triphosphate; TPNH, reduced triphosphopyridine nucleotide.

from chicken liver and the alteration of enzyme levels in vitamin B_{12} deficient chickens are described.

Methods

In the earliest studies, ammonium sulfate fractions (35 to 50 per cent) were prepared from chicken, rat, and pig livers essentially as described by Stevens and Sakami.[10] The protein concentrations ranged between 30 and 40 mg./ml., and usually 1 to 2 mg. of protein were used in the incubations.

The purification of the chicken liver enzyme was done as follows: 40 gm. of chicken liver (commercial source) were homogenized in 2½ volumes of 0.1 *M* Tris buffer, *p*H 7.4. The homogenate was first centrifuged at 5000 × *g* for 5 minutes and then at 105,000 × *g* for 60 minutes. The supernatant fraction was treated with a neutralized saturated solution of ammonium sulfate and the protein that precipitated between 35 and 50 per cent saturation was disolved in 0.01 *M* Tris buffer, *p*H 7.4. The fraction was dialyzed against four liters of 0.005 *M* Tris buffer, *p*H 7.4, and adjusted to a protein concentration of 20 mg./ml. Sixty ml. of the dialyzed fraction were then adsorbed onto calcium phosphate gel at a ratio of 1 mg. gel per mg. protein. Following washing of the gel with 0.01 *M* KPO_4 buffer, *p*H 7.4, enzyme activity was eluted from the gel with 60 ml. of 0.2 *M* KPO_4 buffer, *p*H 7.4. The gel eluate was fractionated with saturated ammonium sulfate, and the precipitate that formed between 30 and 50 per cent saturation was dissolved in 0.01 *M* KPO_4 buffer, *p*H 7.4. This fraction (50 mg. protein per ml.) was dialyzed for eight hours in 0.005 *M* Tris buffer, *p*H 7.4. Three ml. of the dialyzed enzyme solution were diluted to 30 ml. with 0.001 *M* Tris buffer, *p*H 7.4, and applied slowly to a DEAE-cellulose column equilibrated with 0.005 *M* Tris buffer, *p*H 7.4 (1 × 9 cm.). Following preliminary washings with 20 ml. of 0.01 *M* KPO_4 buffer, *p*H 7.4, and 0.01 *M* KPO_4 buffer, *p*H 7.4 containing 0.1 *M* NaCl, the enzyme was eluted with 20 ml. of 0.01 *M* KPO_4 buffer, *p*H 7.4 containing 0.2 *M* NaCl. The DEAE eluate (1.4 mg. protein/ml.) was treated with saturated ammonium sulfate, and the protein that precipitated between 0 and 50 per cent was dissolved in 2 ml. 0.01 *M* Tris buffer, *p*H 7.4 (11 mg. protein per ml.).

CH_3B_{12} was synthesized by the general procedure of Smith *et al.*[7] using CH_3I. $C^{14}H_3B_{12}$ was prepared in the same manner using $C^{14}H_3I$. $C^{14}H_3THF$ was prepared according to the procedure of Keresztesy and Donaldson.[18] Solutions of CH_3THF were prepared in 0.1 *M* mercaptoethanol.

The transfer of the methyl group from $C^{14}H_3THF$ to homocysteine was studied in the following manner: incubations, in a total volume of 0.2 ml., were performed at 37° C. and contained enzyme (0.02 to 0.1 ml.); $C^{14}H_3THF$ (specific activity, 700 cpm/mμmole), 30 mμmoles; vitamin B_{12}, CH_3B_{12} or B_{12} coenzyme, 10 mμmoles; AMe, 5 mμmoles: L-homocysteine (prepared from the thiolactone derivative), 50 mμmoles; ATP, 100 mμmoles; TPNH, 50 mμmoles and KPO_4 buffer, *p*H 7.4, 10 μmoles.

Anaerobic experiments were performed in Thunberg tubes. The incubations were diluted to 0.5 ml. with cold water and passed through a column (½ × 2 cm.) of Dowex 1-Cl. The resin was washed with an additional 1 ml. of H_2O. Both the effluent and wash were collected in a counting vial, and 10 ml. of a naphthalene-dioxane counting fluid were added.[19] The radioactivity was determined in a Packard Tri-carb Scintillation Counter. Under these conditions methionine was found in the column effluent and wash, while the CH_3THF was essentially quantitatively retained by the resin.

The transfer of the methyl group from CH_3B_{12} to homocysteine was assayed in the following manner: incubations were performed in a total volume of 1 ml. containing 0.2 to 0.4 ml. of enzyme, 70 mμmoles of $C^{14}H_3$ B_{12} (specific activity 1800 cpm. mμmole), 250 mμmoles of L-homocysteine and 100 μmoles of Tris chloride buffer, *p*H 7.4, or KPO_4 buffer, *p*H 7.4. The reaction was stopped by the addition of 1 ml. of 0.1 N HCl containing 1 μmole of methionine. The addition of 0.4 ml. of a charcoal suspension (40 mg.) removed most of the CH_3B_{12}. After centrifugation to remove the charcoal, the supernatant fraction was exposed to light to decompose any residual CH_3B_{12}. The solution was applied to a Dowex 50 H^+ column (½ × 3 cm.) and the column washed with 5 ml. of H_2O. The methionine was then eluted with 3 ml. of 3 N NH_4OH and the radioactivity determined as described above. Methionine recovery averaged between 60 and 65 per cent.

That the radioactive product formed in the reactions with $C^{14}H_3THF$ and $C^{14}H_3$ B_{12} was methionine was verified by ascending chromatography of the product (first purified by adsorption and elution from a Dowex 50 H^+ column) on Whatman #1 paper (butanol, acetic acid, H_2O 60:15:25, and ethanol, 1 *M* ammonium acetate, 7.5:3). Essentially all the radioactivity was present in the methionine area with a small amount in the methionine sulfoxide region.

Chicks (Arbor Acres) were made B_{12} deficient using a high fat (24 per cent) diet as described by Spivey Fox *et al.*[20] The animals were sacrificed when four weeks old. The control group of chicks (B_{12} added to the diet) had an average weight of 433 gm. while those on the B_{12} deficient diet averaged 184 gm. Each group contained 8 to 10 animals. A similar study employed a low-fat diet (4 per cent) as described by Spivey Fox *et al.*[20] In this series the control group had an average weight of 470 gm. while the B_{12} deficient animals averaged 320 gm. High speed supernatant fractions were prepared according to the procedure of Stevens and Sakami.[10] To compensate for the large weight differences in the various groups of animals, enzyme activity is described in terms of specific activity (mμmoles methionine formed per 30 minutes per mg. of protein).

Results

Although methionine biosynthesis from CH_3THF was observed in several animal extracts, chicken liver was selected as source material for purifi-

cation because of the background of nutritional data obtained with this animal.[14,16,20] The procedure for enzyme purification is described under *Methods* and a summary of a sample procedure is presented in TABLE 1. A range in the overall purification from 10 to 30 fold was obtained

TABLE 1

PURIFICATION OF CHICKEN LIVER ENZYME

	Specific activity*	% recovery
1. 105,000 × *g* supernatant fraction	0.27	—
2. Ammonium sulfate, 35 to 50 per cent fraction, dialyzed (AS I)	0.54	35
3. Calcium phosphate gel eluate	1.5	32
4. Ammonium sulfate, 30 to 50 per cent fraction, dialyzed (AS II)	2.0	19
5. DEAE-cellulose eluate	8.2	19
6. Ammonium sulfate, 0 to 50 per cent fraction, (AS III)	8.7	12

* mμ*M* methionine formed per 30 minutes per mg. protein.

among different preparations by this procedure. TABLE 2 shows the requirements for methyl transfer from CH_3THF to homocysteine with AS III (see TABLE 1) as the enzyme. A striking dependence on vitamin B

TABLE 2

METHIONINE FORMATION FROM CH_3THF

	Methionine mμmoles formed/30 min.	
	Aerobic	Anaerobic
Complete	2.15	7.2
−AMe	0.16	0.75
$-B_{12}$	0.1	5.4
−Homocysteine	0.21	0.48
−TPNH	2.2	6.6
−ATP	2.3	6.9

* Incubation conditions described in text. An AS III preparation was used as enzyme source.

is observed under aerobic conditions, but not anaerobically. In addition the reaction requires AMe and, the methyl group acceptor, homocysteine aerobically and anaerobically. TPNH and ATP which stimulated the reaction slightly in earlier fractions (AS I) were without significant effect with AS III.

The aerobic response to vitamin B_{12} is similar to the stimulation observed with cobamide compounds in the phosphoroclastic reaction.[21]

Here, too, the cobamide effect was not observed under anaerobic conditions, and evidence was presented that the cobamides functioned by maintenance of sulfhydryl groups on the enzyme in a reduced state.[22]

The cobamide requirement observed under aerobic conditions, but not anaerobically, was not unique with the chicken enzyme but was also observed with extracts of rat and pig liver (TABLE 3). The cobamide requirement seen with all the extracts tested could be satisfied by either vitamin B_{12}, CH_3 B_{12}, or B_{12} coenzyme. AMe and homocysteine were required both aerobically and anaerobically in all systems. With *E. coli* extracts, Guest *et al.*[8] have shown that CH_3 B_{12} can transfer its methyl group to homocysteine. Although there is no evidence for an anaerobic cobamide requirement in the animal systems, a transfer of the methyl group from CH_3 B_{12} to homocysteine has been demonstrated with extracts of liver. Methyl transfer from CH_3 B_{12} required only enzyme and homocysteine; there was no effect of AMe or CH_3THF. As shown in TABLE 4, a nine-fold purification of the enzyme that catalyzed CH_3 B_{12} transfer was obtained from chicken liver using the same procedure that resulted in a

TABLE 3

EFFECT OF VITAMIN B_{12} ON METHIONINE FORMATION FROM CH_3THF AEROBICALLY AND ANAEROBICALLY

	Methionine mμmoles formed per 30 min.			
	Rat		Pig	
	Aerobic	Anaerobic	Aerobic	Anaerobic
Complete system	6.6	7.4	4.0	4.5
−vitamin B_{12}	0.41	6.2	1.1	6.0
−AMe	0.37	0.24	0.5	0.1

Incubation conditions are described in the text. An AS I preparation was used in these studies.

TABLE 4

METHIONINE FORMATION FROM CH_3 B_{12} WITH CHICKEN LIVER FRACTIONS

Fraction	Methionine mμmoles per 2 hr. per mg. protein
High speed supernatant	0.28
AS I	0.33
AS III	2.58

Details of the incubation and assay are described under *Methods*. The incubations contained 0.3 ml. of each enzyme fraction. High speed supernatant, 62 mg./ml.; AS I, 93 mg./ml.; AS III 49 mg./ml.

10-fold purification of the enzyme that catalyzed methyl transfer from CH_3THF to homocysteine. A transfer of the methyl group from CH_3 B_{12} to homocysteine has also been observed in extracts of rat and pig liver, although to a lesser degree.

Another approach to understanding the role of vitamin B_{12} in methionine biosynthesis in animals is by studying the effect of nutritional deficiency. As described in the section on Methods, groups of chicks were deprived of vitamin B_{12} while on diets of known fat content. There was little synthesis of methionine from CH_3THF in preparations obtained from these animals (TABLE 5). However, preparations from control animals

TABLE 5

ALTERATION IN ENZYME LEVEL* WITH VITAMIN B_{12} DEFICIENCY

Diet	Methionine mμmoles formed per 30 min. per mg. protein
Low fat + B_{12}	1.51
Low fat − B_{12}	0.13
High fat + B_{12}	1.61
High fat − B_{12}	0.10

* Transfer of the methyl group from CH_3THF to homocysteine. Each group contained eight animals. Each figure is the average value obtained from at least five individual animals.

(with B_{12} added to the diet) were highly active, having 12 to 16-fold greater specific activities. The marked difference between control and vitamin B_{12} deficient extracts was also apparent when the assays were performed under anaerobic conditions. Attempts to restore the *in vitro* activity in vitamin B_{12} deficient extracts with vitamin B_{12}, CH_3 B_{12}, B_{12} coenzyme, or B_{12r} were unsuccessful. Although previous studies[16] reported that a high fat diet had a synergistic effect on the development of B_{12} deficiency in chickens, this was not observed with respect to the enzyme activity in the present study. As can be seen in TABLE 5 levels of fat intake had little effect on methyl transfer from CH_3THF to homocysteine.

Discussion

The significance of a cobamide stimulation, which is present under aerobic but not under anaerobic conditions, is difficult to ascertain at this point in our investigation. One explanation is that the cobamides maintain essential sulfhydryl groups on the enzyme in a reduced state. Peel has indicated that a similar effect occurs, at very low concentrations, in the phosphoroclastic reaction.[22] Other workers have reported marked alterations in sulfhydryl compounds during vitamin B_{12} deficiency in different species.[23-28]

The possibility exists that an anaerobic dependence on vitamin B_{12} will

develop upon further resolution of the purified enzyme, as was the case with mammalian methylmalonyl isomerase.[29] If a methyl B_{12} protein is an intermediate in the terminal reaction in methionine synthesis,[8] the ability of the liver extracts to transfer the methyl group of CH_3 B_{12} to homocysteine may be one indication that vitamin B_{12} is involved in the formation of methionine in animals. The marked lowering of enzymatic activity for methyl transfer from CH_3THF to homocysteine in B_{12} deficient chicks is further evidence of a cobamide effect on methionine synthesis. However, restoration of activity *in vitro* could not be obtained by the addition of vitamin B_{12} or its derivative to the enzymatic system. Preliminary observations have indicated that short-term dietary replenishment of vitamin B_{12} leads to partial restoration of enzyme activity. Norohna and Silverman[30] observed that during vitamin B_{12} deficiency in animals, there was an accumulation of prefolic acid (CH_3THF) and a correspondent reduction of the N-5 formyl, N-10 formyl, and other N-10 derivatives of tetrahydrofolic acid. The marked depression in the enzyme responsible for transfer of the methyl group from CH_3THF to homocysteine would be consistent with the observed accumulation of methylated folate derivatives, as well as the reduction in the levels of reduced derivatives of folate. It is also known that methionine administration results in a reduction of CH_3THF levels in liver with an increase in the levels of formylated THF derivatives.[30] Vitamin B_{12} has a similar effect although to a much lesser degree. These same workers[30] in attempting to explain these effects postulated that a methyl acceptor formed from the dietary methionine accepts the methyl groups from CH_3THF. An alternative explanation is that methionine or some derivative of methionine might exert a feedback inhibition on the enzymes required for the synthesis of CH_3THF.

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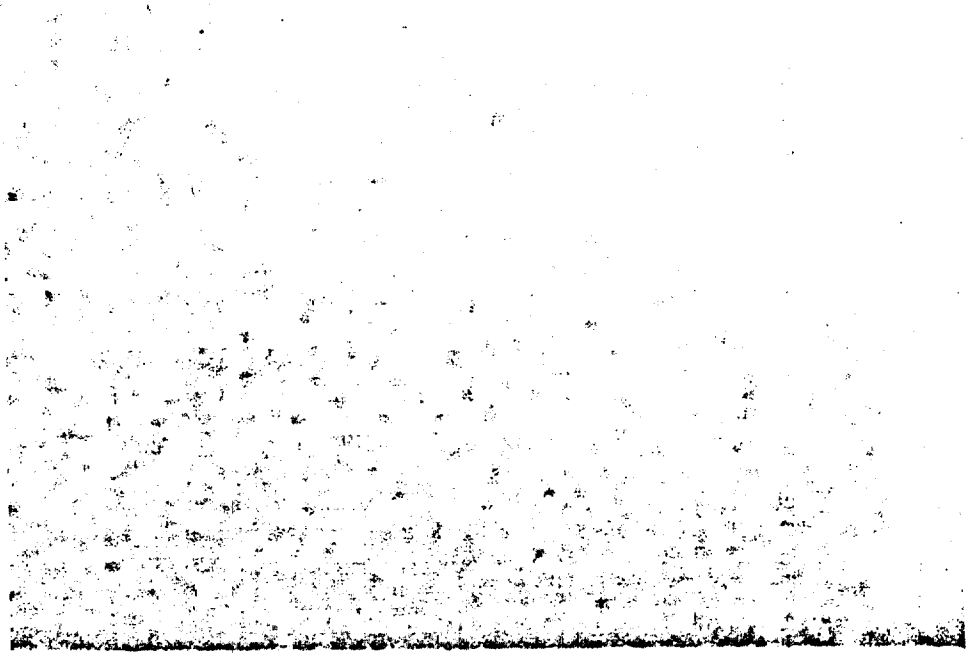

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Effect of Dietary Vitamin B_{12} on Methionine Biosynthesis by Chick Liver Homogenates.* (26816)

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It was observed several years ago that dietary vitamin B_{12} would spare the methyl requirements for animals(1). More recently, considerable evidence has accumulated to implicate Vit. B_{12} in methionine biosynthesis in bacterial systems(2,3). Arnstein(4) has demonstrated in *in vivo* experiments with rats that Vit. B_{12} stimulates the conversion of formate to the methyl groups of choline and methionine. We have reported(5,6) that Vit. B_{12} stimulates the conversion of formate to the 5-methyl group of thymine by chick bone marrow cells but does not stimulate conversion of formaldehyde to the 5-methyl group of thymine. These results suggest that Vit. B_{12} functions in reduction of one-carbon compounds between the formate and formaldehyde levels of oxidation. In the present experiments we have studied the effect of Vit. B_{12} deficiency in chicks on formation of the methyl group of methionine by liver homogenates and the results suggest that B_{12} stimulates the conversion of formate to methionine methyl but does not increase conversion of formaldehyde to methionine methyl, a situation analogous to our previously reported results in thymine formation by chick bone marrow cells.

Experimental. Day-old White Leghorn chicks were given the Vit. B_{12} and methionine-deficient basal diet described by Spivey-Fox *et al.*(7). When present, methionine was added at a concentration of 6% of the diet and Vit. B_{12} was given by weekly injections at a dosage of 3 μg per chick the first week and 9 μg per chick thereafter. After 3 to 4 weeks on the various diets, the chicks were taken for experiment. Peripheral blood counts were made using standard hematological procedures.

Methionine formation from homocysteine and formate-C^{14} or formaldehyde-C^{14} was determined in liver homogenates. The livers were homogenized in an all-glass homogenizer with 2 volumes of Robinson's buffer(8). The incubation mixture consisted of 2 ml of homogenate, 3 mg of DL-homocysteine, and either 0.27 μmole of formate-C^{14} (142,000 c.p.m.) or 2 μmoles of formaldehyde-C^{14} (140,000 c.p.m.). The final volume was 2.5 ml. After an hour of incubation at 37° under air in the Dubnoff shaker, the reaction was stopped by addition of 0.5 ml of 50% trichloroacetic acid and the contents of the incubation beakers were transferred to 12 ml conical centrifuge tubes and centrifuged. 1.5

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TABLE I. Effects of Dietary Supplements of Methionine and Vitamin B_{12} on Growth and Hemogram of Chicks.

Supplement	Weight gain, g/4 wk	Erythrocytes, millions/μl	Hemoglobin, g/100 ml	Hematocrit, %
None	154	2.83	9.6	29
Methionine	241	2.70	9.1	29
B_{12}	237	2.83	9.5	29
Methionine + B_{12}	250	3.04	9.7	31

ml of the supernatant solution was taken and 200 mg of carrier L-methionine added and dissolved with heating. The methionine was precipitated on cooling with addition of 95% ethanol and recrystallized in this fashion a total of 6 times. The methionine was then plated on aluminum planchets and counted at infinite thickness with an end-window Geiger counter. The results are reported as counts per minute of the infinite thickness samples. To correct for any non-enzymatic reaction between formaldehyde, formate and homocysteine zero time samples were taken and the counts obtained subtracted from the counts obtained in the incubated samples. Five chicks from each group were used in these experiments.

Results and discussion. Supplements of either B_{12} or methionine stimulated growth of chicks as indicated by the data in Table I. In the presence of adequate methionine B_{12} produced no significant effect on the growth rate. These data again point out the well-known effect of Vit. B_{12} in sparing the methyl requirements of animals. The hemogram of the chicks was not significantly influenced by B_{12} deprivation indicating the mildness of the B_{12} deficiency.

TABLE II. Influence of Dietary Vitamin B_{12} and Methionine on Formation of Methionine Methyl by Chick Liver Homogenates. Results reflect total C^{14} incorporated into methionine. See text for details of calculation.

Supplement	C^{14} substrate employed: Formate	Formaldehyde
None	239*	43†
B_{12}	511	12
Methionine	237	44
Methionine + B_{12}	353	25

* B_{12} effect statistically significant. $P < .01$. Methionine effect not statistically significant.
† B_{12} effect statistically significant. $P < .05$. Methionine effect not statistically significant.
Statistically treated by analysis of variance.

Vit. B_{12} stimulated the conversion of formate to methionine methyl but appeared to inhibit conversion of formaldehyde to methionine methyl as indicated by the data in Table II. This observation is in agreement with studies of the influence of Vit. B_{12} on biosynthesis of the methyl group of thymine (5,6). Methionine supplementation did not exert a statistically significant effect on methionine biosynthesis by liver homogenates.

These results again demonstrate the involvement of Vit. B_{12} in methyl synthesis by animals and substantiate our earlier suggestions concerning the site of action of B_{12}. The results indicate that Vit. B_{12} functions between the formate and formaldehyde levels of oxidation, a reaction catalyzed by the enzyme methylene tetrahydrofolic dehydrogenase. We have previously reported a reduction in activity of this enzyme in tissues from B_{12} deficient animals(9).

Summary. Liver homogenates from B_{12}-deficient chicks converted formate to methionine methyl at a reduced rate and converted formaldehyde to methionine methyl at an accelerated rate. These results suggest the involvement of Vit. B_{12} in reduction of formate to formaldehyde, a reaction mediated by methylene tetrahydrofolic dehydrogenase.

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Intrinsic Factor–mediated Attachment of Vitamin B_{12} to Brush Borders and Microvillous Membranes of Hamster Intestine *

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Summary. Hamster intrinsic factor (IF) preparations markedly enhanced the uptake of ^{57}cobalt-labeled cyanocobalamin (B_{12}-^{57}Co) by brush borders and microvillous membranes isolated from villous absorptive cells obtained from the distal but not the proximal half of hamster intestine. A similar effect was observed with rat and rabbit IF preparations, but IF preparations obtained from man, dog, and hog were ineffective. After fractionation of hamster IF preparations by gel filtration or ion exchange chromatography, the extent to which each fraction enhanced B_{12}-^{57}Co uptake by brush borders correlated closely with the vitamin B_{12} binding capacity of the fraction. IF-mediated attachment of B_{12}-^{57}Co to brush borders occurred rapidly, was not diminished by removal of glucose or oxygen from the incubation medium, and was not significantly altered when incubation temperatures were reduced from 37° C to 7° C. Marked reduction in uptake occurred, however, in the absence of divalent cations.

IF enhanced B_{12}-^{57}Co uptake by brush borders isolated from the proximal half of the intestine when these proximal brush borders were preincubated with supernatant fluid obtained after centrifugation of homogenates of distal intestinal mucosa at 28,500 *g*. The factor in this supernate responsible for the effect on proximal brush borders was shown to be particulate in nature upon centrifugation at speeds of 54,500 *g* or greater. The resultant pellet contained ribosomes and membranous fragments.

Prolonged incubation of brush borders with crude saline extracts of hamster gastric mucosa resulted in decreased uptake of B_{12}-^{57}Co and marked lysis of brush borders with concomitant release of tissue nitrogen. Neither lysis of brush borders nor decreased uptake of B_{12}-^{57}Co with prolonged incubation was observed when hamster IF was partially purified. Furthermore, uptake of B_{12}-^{57}Co by brush borders increased with increasing purity of the IF preparation used.

These results demonstrate IF-mediated attachment of B_{12}-^{57}Co to brush borders and microvillous membranes of hamster intestinal cells and provide further support for the presence of a specific receptor for IF-bound vitamin

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B_{12} at the microvillous surface of the intestinal cell. IF-mediated attachment to the intestinal cell surface appears to be facilitated by divalent cations and to result from adsorption rather than an energy-requiring enzymatic reaction. Crude sources of hamster IF contain a factor which causes lysis of brush borders *in vitro* and which may explain in part the inhibitory effects of IF excess previously observed *in vitro*.

Introduction

Intrinsic factor (IF), secreted by the stomach, is required for efficient absorption of physiological quantities of vitamin B_{12} from the distal intestine (2). The mechanism by which IF promotes absorption of the vitamin is unknown, but it is generally accepted that vitamin B_{12} must be bound by IF to form a macromolecular complex if efficient absorption is to occur (3). IF enhances uptake of vitamin B_{12} *in vitro* by everted sacs (4, 5), rings (6), and homogenates (7) of intestine obtained from a variety of mammalian species including man (8). Such *in vitro* studies, however, do not localize the cellular sites of IF action and do not distinguish transfer of the vitamin through the cell membrane from uptake by the membrane itself. Although there is evidence to suggest a specific intestinal receptor for the IF-vitamin B_{12} complex (5), such a receptor has not been isolated or localized to the surface of the intestinal absorptive cell.

This report describes studies of the effect of IF on the uptake of ^{57}cobalt-labeled cyanocobalamin (B_{12}-^{57}Co) by brush borders and microvillous membranes isolated from absorptive cells of hamster intestine. The results provide further support for the presence of a specific receptor for the IF-vitamin B_{12} complex at the microvillous surface of the intestinal cell. In addition, crude sources of hamster IF were shown to contain a factor that causes lysis of isolated brush borders.

Methods

Isolation of brush borders and microvillous membranes. Procedures similar to those previously described by Miller and Crane (9) and Eichholz and Crane (10) were used to isolate brush borders and microvillous membranes. Golden hamsters weighing 100 to 150 g were sacrificed by a blow on the head. The entire small intestine was immediately flushed *in situ* with cold 0.15 M NaCl, excised, divided into proximal and distal halves, opened, and spread out on wet paper towels with the mucosal surface facing upwards. The mucosal surface was scraped lightly with a glass slide to collect villous cells. Scrapings pooled from either the proximal or distal half of the intestines of six hamsters were placed in 100 ml of cold 5 mM EDTA solution buffered to pH 7.4. Homogenization was carried out for 25 seconds in a Waring blendor; the speed of the blendor was controlled by a rheostat. Since homogenization was found to be a critical step in preparing satisfactory brush border fractions, several preliminary experiments were necessary to determine the optimal speed and duration of homogenization. The homogenate was then filtered through no. 25 bolting silk[1] to remove mucus and large particles. The entire procedure from the time the animals were sacrificed through recovery of the filtered homogenate was carried out in a cold room at 6° C.

The filtrate was then centrifuged in an International PR-2 refrigerated centrifuge for 10 minutes at 1,500 rpm. Thereafter, the sediment was washed twice with 50 to 60 ml of cold 5 mM EDTA and centrifuged again at 1,500 rpm. The pellet was then suspended in Krebs-Ringer bicarbonate solution (KRB) at pH 7.4, centrifuged at 1,500 rpm, and resuspended in cold KRB. This suspension was centrifuged at 500 rpm for 1 minute, and the sediment containing clumped material and nuclei was discarded. Although this step substantially reduced the yield of brush borders, it provided much purer preparations as evaluated by light microscopy. The supernate was then centrifuged at 2,500 rpm for 10 minutes to yield a pellet of relatively pure brush borders.

Brush borders thus prepared from either the proximal or distal half of the intestine of 6 hamsters (4.0 to 7.5 mg of tissue protein) were further fractionated to isolate microvillous membranes (10). The centrifuged pellet of freshly prepared brush borders was suspended in 5 ml of cold 1.0 M Tris buffer (pH 7.0), shaken vigorously with a Vortex mixer for 3 minutes, and allowed to stand in the cold for 30 to 90 minutes. The resulting suspension was layered at the top of a discontinuous density gradient consisting of 20, 30, 40, 50, and 60% glycerol and centrifuged at 63,000 *g* for 10 minutes in a Spinco model L preparative ultracentrifuge. This consistently resulted in the separation of particles into A, B, C, C′, and D bands as described by Eichholz and Crane (10). The C and C′ bands containing microvillous membranes were collected by aspiration, washed in an excess of cold KRB, and centrifuged in a Sorvall RC-2 high speed centrifuge at 28,000 *g* for 20 minutes.

Mucosal scrapings pooled from either the proximal or

[1] Obtained from Tobler, Ernst, and Trabler, New York, N. Y.

distal half of 3 hamster small intestines were homogenized for 1 minute in 100 ml of KRB. The whole intestinal mucosal homogenate was then centrifuged at 1,500 rpm for 10 minutes at 4° C, and the pellet was resuspended in KRB.

Light and electron microscopy. Light and electron microscopy was used to evaluate the tissue preparations. A drop or two of brush border suspension was frequently examined with the phase microscope to assess the purity of the suspension before its utilization for biochemical studies.

For detailed evaluation of the purity and morphologic preservation of brush borders, 5 ml of chilled chromeosmium tetroxide (11) was added to pellets that had been isolated as described above. After 5 minutes, the pellets were broken into pieces of approximately 1 mm^3 in the fixative solution. After 1 to 2 hours of fixation, the fragments were transferred to glass weighing bottles and fixed again in 10% neutral isotonic formal for 1 hour, rapidly dehydrated in graded strengths of ethyl alcohol, and embedded in epoxy resin by the method of Luft (12). The microvillous membranes were fixed, dehydrated, and embedded in the same fashion, except that the entire procedure including embedment was carried out without disruption of the pellet in the tube originally used for recovery of the membranes. Thus, the orientation of the membrane pellet was maintained. Pieces of the embedded microvillous membrane pellets were cut out of the Epon block and mounted with epoxy cement on short aluminum rods machined to fit the microtome chuck after they had been oriented so that the full thickness of the pellets could be sectioned and systematically studied from top to bottom. Sections of both brush border and microvillous membrane pellets were cut 1 μ thick with glass knives, mounted on glass slides, and stained for light microscopic study with toluidine blue (13) or the periodic acid–Schiff technique.

Thin sections for electron microscopy were cut with diamond knives, mounted on carbon-coated copper mesh grids, and doubly stained with uranyl acetate (14) and lead citrate (15). Stained sections were studied with an RCA EMU-3G electron microscope.

Intrinsic factor preparations. IF preparations were obtained from a variety of animal species. Hamster and rat gastric juice was collected by ligation of the pylorus of lightly anesthetized, fasting animals and instillation of 1 ml of 10% $NaHCO_3$ through a fine polyethylene tube into the stomach. After 5 hours the animals were sacrificed, the stomachs were opened, and gastric contents of 6 to 12 animals were pooled. The pH of this *in vivo* neutralized gastric juice varied from 7.5 to 8.5. After centrifugation at 2,500 rpm and 4° C for 30 minutes, the supernatant gastric juice was titrated to pH 7.0 with 0.1 N HCl and stored at − 20° C until used.

Human gastric juice was collected from fasting subjects after injection of 1.5 mg of histalog per kg body weight. Specimens were collected in iced containers and, in order to inactivate pepsin (16), were immediately titrated first to pH 10 with NaOH, and then to pH 7.0 with HCl.

Saline extracts of hamster, rat, rabbit, and dog gastric mucosa were prepared from mucosal scrapings of stomach obtained immediately after animals had been sacrificed. The scrapings were homogenized in Potter-Elvejhem glass homogenizers, and the homogenate was then centrifuged at 2,500 rpm and 4° C for 10 minutes. The supernate was removed and stored at − 20° C until used.

Neutralized hamster gastric juice and saline extracts of hamster gastric mucosa were concentrated by ultrafiltration (17) and fractionated on columns of Sephadex G-200 eluted with 0.05 M phosphate buffer, pH 7.5. In addition, hamster gastric mucosal extracts were chromatographed on columns of Amberlite CG-50 ion exchange resin as previously described by Chosy and Schilling (17). Columns were eluted at 6° C and eluates were collected in 4-ml aliquots by means of an automatic fraction collector.

The vitamin B_{12} binding capacity of the various IF preparations was determined by dialysis. To serial dilutions of an aliquot of each preparation was added 10 nanograms (10^{-9}g, ng) of B_{12}-^{60}Co (SA 1 mc per mg). After standing for 30 minutes at room temperature, 2-ml aliquots were dialyzed at 6° C against two changes of 3 L of 0.15 M NaCl for 48 hours in Visking cellophane bags. After dialysis 1 ml of fluid was removed from each bag, and its radioactivity was compared with that of a 1-ml undialyzed aliquot. After the binding capacity had been calculated, the concentration of the IF preparation was

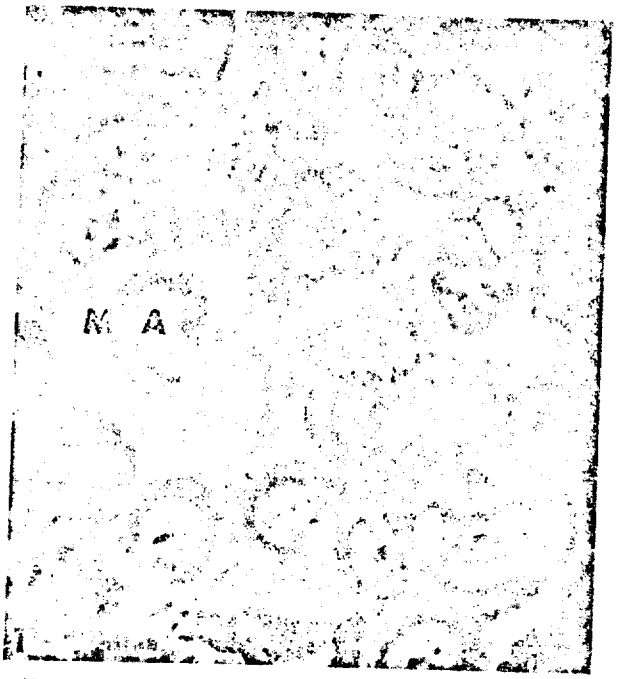

FIG. 1. LIGHT MICROGRAPH OF A SECTIONED PELLET OF HAMSTER BRUSH BORDERS. The preparation consists almost exclusively of brush borders. The microvillous surface (M) and underlying apical cytoplasm (A) can be readily identified. Epon embedment, toluidine blue, × 2,000.

diluted to the desired level by the addition of 0.15 M NaCl.

Nitrogen concentrations were determined by a micro-Kjeldahl technique (18), and pepsinogen activity was assayed by the hemoglobin substrate method of Anson and Mirsky (19).

Uptake of B_{12}-^{57}Co by brush borders and microvillous membranes. Freshly prepared pellets containing brush borders or microvillous membranes were resuspended in cold KRB, and 1-ml aliquots of these suspensions were added to 25-ml Erlenmeyer flasks containing 4 ml of KRB, 2 ng of B_{12}-^{57}Co (SA 13.5 to 20 μc per μg), and sufficient IF preparation to bind the 2 ng of cyanocobalamin. In control flasks 0.15 M NaCl was added instead of an IF preparation. Incubations were usually performed with KRB containing 250 mg per 100 ml glucose at 37° C in room air for 60 minutes in a Dubnoff shaking metabolic incubator. Some incubations were carried out in an atmosphere of 95% O_2 and 5% CO_2; in other experiments the flasks were stoppered after they had been thoroughly gassed with nitrogen. The brush borders added to each flask usually contained 600 to 800 μg of tissue protein, but readily interpretable results could be obtained with as little as 50 to 100 μg of tissue protein. Microvillous membrane preparations added to incubation mixtures contained 100 to 200 μg of tissue protein.

Immediately after incubation an excess of cold KRB was added and the mixture was centrifuged at 4° C and 2,500 rpm for 10 minutes. The tissue pellet was washed twice more in cold KRB and centrifuged again. Radioactivity remaining in the washed pellet was determined in a Packard Autogamma detector. Background activity did not exceed 50 cpm; each picogram of B_{12}-^{57}Co yielded 20 to 30 cpm above background. Tissue uptake was calculated as picograms B_{12}-^{57}Co per milligram tissue nitrogen.

Results

Purity and integrity of tissue preparations. Figure 1 is a light micrograph of a representative field of a sectioned hamster brush border pellet fixed in osmium tetroxide, embedded in Epon, and stained with toluidine blue. The pellets consisted of pure brush borders, except for an occasional isolated nucleus and a small amount of amorphous material presumably derived from epithelial cell cytoplasm. The isolated brush borders could be readily identified by their densely stained, crescent-shaped, microvillous surface and by the paler underlying attached apical cytoplasm.

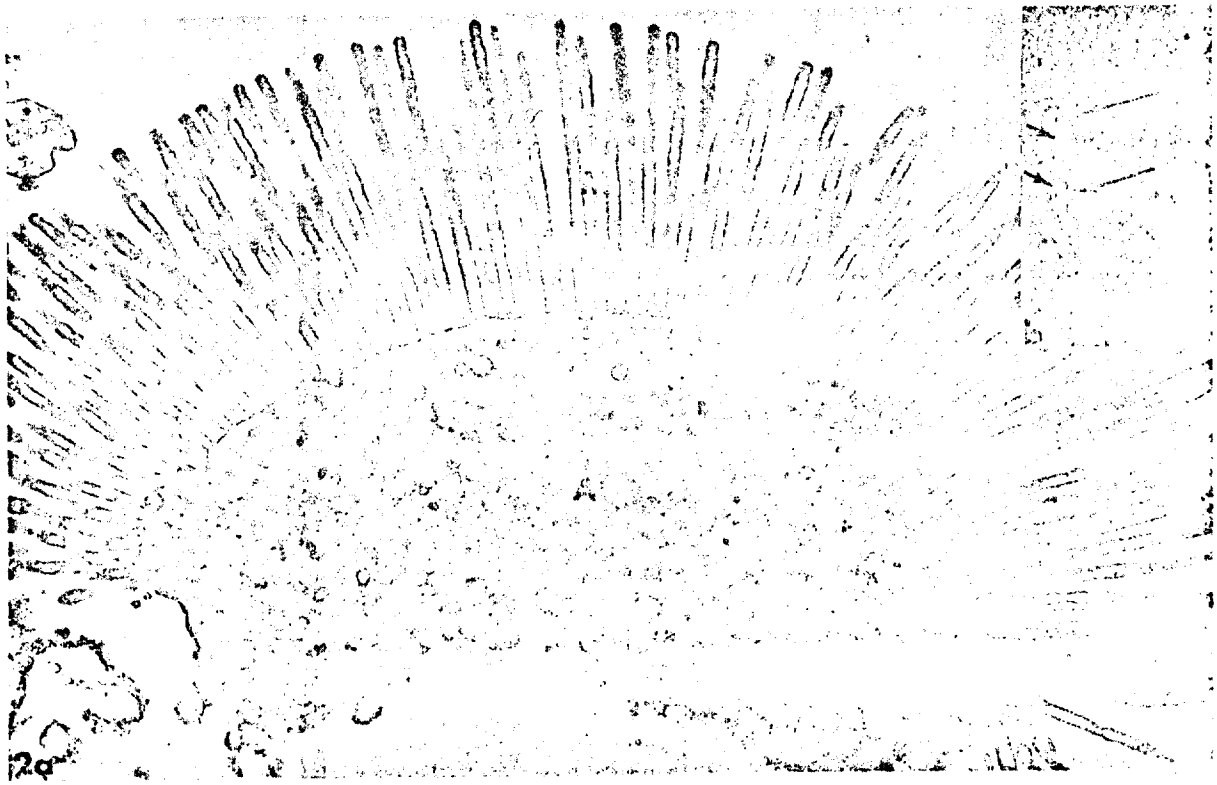

FIG. 2. FINE STRUCTURE OF A TYPICAL HAMSTER BRUSH BORDER. *a*) Electron micrograph demonstrating good morphologic preservation of the microvilli (M), terminal web region (T), and attached apical cytoplasm (A). × 17,000. b) High magnification of a portion of a microvillus from an isolated brush border. The trilaminar structure of the microvillous membrane (long arrows) as well as its surface coat (short arrows) is well preserved. × 110,000.

The structural features of the microvilli of isolated hamster brush borders were not greatly altered by the fractionation procedure. Figure 2a is an electron micrograph of a typical isolated brush border. The microvilli, terminal web, and the small portion of attached apical cytoplasm can be readily identified and are well preserved. At higher magnification, it is apparent that the surface coat of the microvillous membrane is more compact than it is in intact, well-preserved hamster epithelium, but the trilaminar unit membrane structure of the apical plasmalemma and the microvillous core are well preserved (Figure 2b).

The microvillous membrane pellets isolated from Tris-disrupted brush borders also appeared remarkably pure and morphologically well preserved (Figure 3a). Both longitudinal and cross sections of the narrow membranous sleeves of the microvilli were seen; some wider membranous profiles that probably resulted from vesiculation of the membrane during the isolation procedure were also present. The characteristic trilaminar structure of the microvillous membrane with at least a portion of the surface coat still attached to its outer lamina could be regularly identified at higher magnification (Figure 3b).

Effect of intrinsic factor on B_{12}-^{57}Co uptake by tissue preparations. As shown in Figure 4, neutralized hamster gastric juice consistently enhanced attachment of B_{12}-^{57}Co to brush borders and microvillous membranes obtained from the distal but not the proximal half of hamster small intestine. In the presence of IF uptake by distal brush borders was fivefold greater and by distal microvillous membranes was tenfold greater than was uptake by whole mucosal homogenates. In contrast, uptake of B_{12}-^{57}Co by whole mucosal homogenates, brush borders, and microvillous membranes ob-

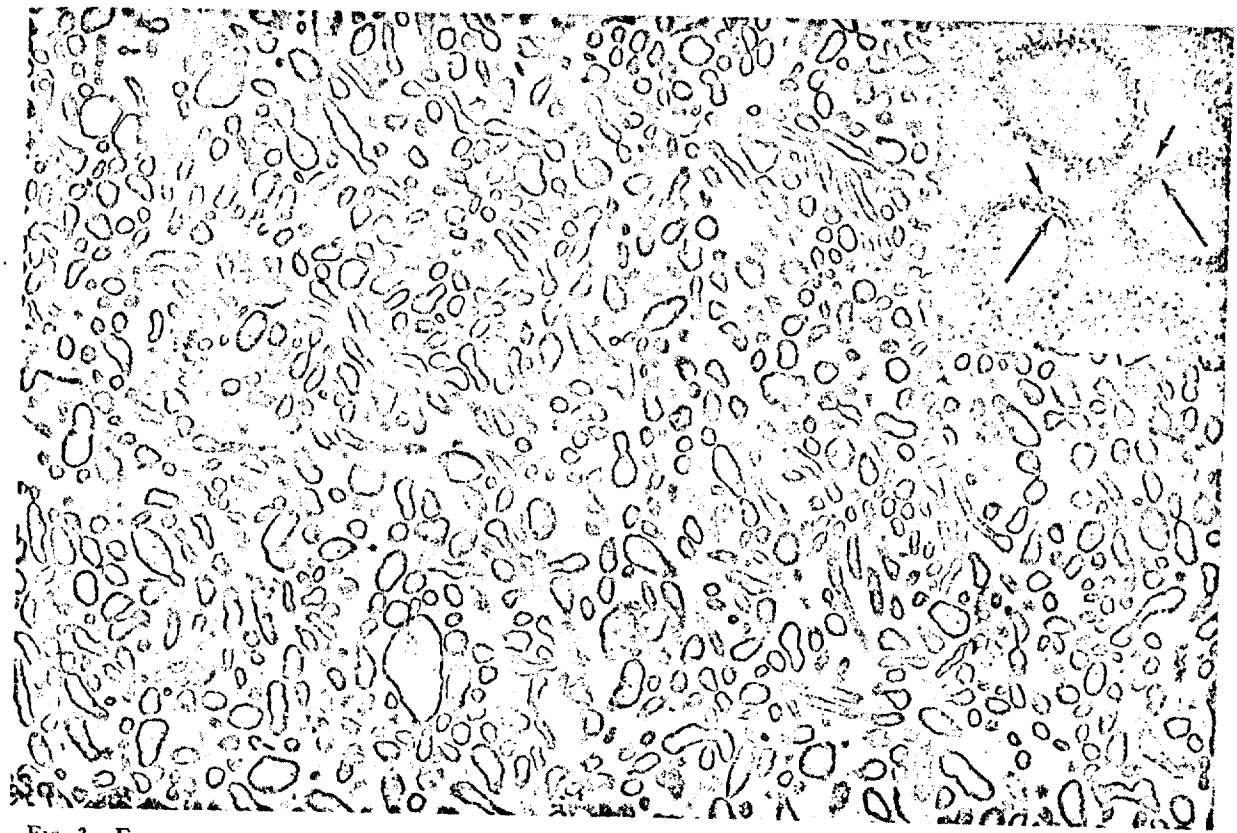

FIG. 3. FINE STRUCTURE OF A TYPICAL PELLET OF ISOLATED MICROVILLOUS MEMBRANES. a) Survey electron micrograph illustrating the purity of the preparation. Longitudinally sectioned, tangentially sectioned, and cross sectioned profiles as seen. Some of the membranous profiles are larger in diameter than well-formed microvilli. This probably represents vesiculation of some of the microvilli during the preparative procedure. × 15,000. b) High magnification of isolated microvillous membranes. Their trilaminar membrane (long arrows) and surface coat (short arrows) are readily identified. × 110,000.

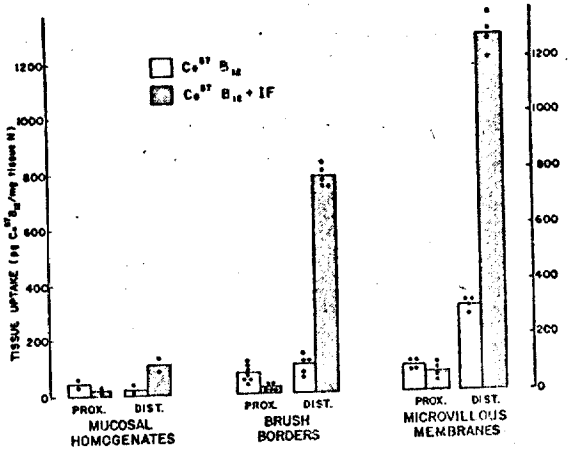

FIG. 4. UPTAKE OF FREE AND INTRINSIC FACTOR (IF)-BOUND B_{12}-^{57}Co BY VARIOUS PREPARATIONS OF HAMSTER INTESTINE. The same pool of neutralized hamster gastric juice was used as IF for all experiments. Dots represent results of individual experiments; bars indicate mean values. IF stimulated uptake of B_{12}-^{57}Co by preparations obtained from the distal but not the proximal half of hamster intestine.

tained from proximal intestine was consistently inhibited by the presence of IF.

Table I summarizes the effects of IF and non-IF binders of vitamin B_{12} obtained from various animal species on B_{12}-^{57}Co uptake by brush borders isolated from distal hamster intestine. Gastric juice or saline extracts of gastric mucosa or both obtained from hamsters, rats, and rabbits enhanced B_{12}-^{57}Co uptake by hamster brush borders, whereas human, hog, and dog IF preparations were without effect. Non-IF B_{12} binders in human saliva and serum and in saline extracts of hamster liver, kidney, and spleen failed to promote uptake of radioactivity by hamster brush borders.

TABLE I

Specificity of intrinsic factor (IF) action on uptake of B_{12}-^{57}Co by hamster brush borders

B_{12}-binding protein*	No. experiments	B_{12}-^{57}Co uptake ± SD
		pg/mg tissue N
None	7	88 ± 15
IF preparations		
Hamster gj†	5	783 ± 68
Hamster gme‡	5	285 ± 23
Rat gj	3	1,064 ± 89
Rat gme	2	392 ± 36
Rabbit gme	2	175 ± 14
Human gj	4	44 ± 18
Hog gme	2	87 ± 6
Dog gme	2	32 ± 15
Non-IF binders		
Human saliva	3	45 ± 4
Human serum	3	28 ± 3
Hamster liver extract	2	61 ± 8
Hamster kidney extract	2	81 ± 11
Hamster splenic extract	2	28 ± 2

* A quantity of each preparation sufficient to bind 2 nanograms (10^{-9} g, ng) B_{12}-^{57}Co was added to incubation flask.
† gj = neutralized gastric juice.
‡ gme = saline extract of gastric mucosa.

As shown in Figure 5, when hamster gastric juice was subjected to dextran gel filtration on Sephadex G-200, a single peak of vitamin B_{12} binding activity was observed in the eluted fractions. When 0.1-ml aliquots of these fractions were incubated with distal brush borders, enhancement of tissue uptake of B_{12}-^{57}Co correlated closely with the vitamin B_{12} binding capacity of the fractions.

Saline extracts of hamster gastric mucosa fractionated on a column of Amberlite CG-50 (Figure 6) yielded 2 peaks of vitamin B_{12} binding activity, the second much larger than the first. Marked enhancement of B_{12}-^{57}Co uptake by hamster brush borders was produced by 0.1-ml aliquots of fractions eluted in the second but not the first peak of vitamin B_{12} binding activity. Pepsinogen activity of gastric mucosal extracts was readily separated from IF activity by chromatography on Amberlite CG-50.

To determine whether IF could attach to brush borders in the absence of vitamin B_{12} we performed the experiment summarized in Table II.

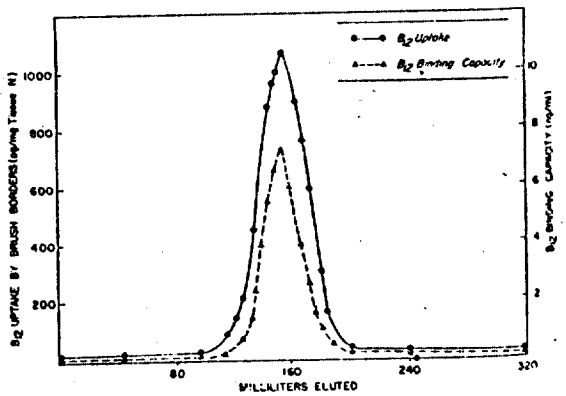

FIG. 5. GEL FILTRATION OF NEUTRALIZED HAMSTER GASTRIC JUICE ON SEPHADEX G-200. Elution with 1 M phosphate buffer, pH 7.4. Eluate collected in 4-ml fractions. Aliquots of the same fraction were tested for B_{12} binding capacity and for effect on uptake of B_{12}-^{57}Co by distal brush borders.

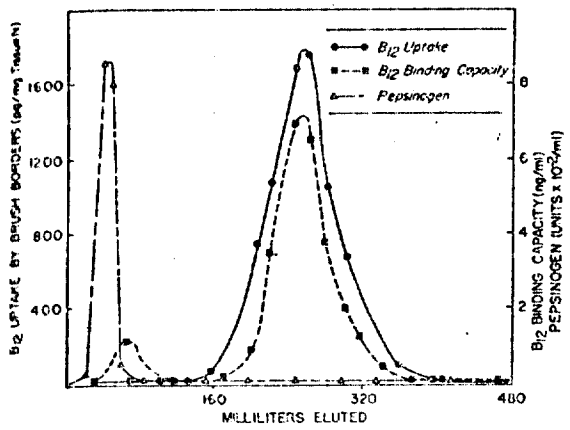

FIG. 6. ION EXCHANGE CHROMATOGRAPHY OF SALINE EXTRACT OF HAMSTER GASTRIC MUCOSA. Elution with 0.58 M acetate buffer, pH 5.4. Aliquots of 4-ml fractions were tested for B_{12} binding capacity, effect on B_{12} uptake by hamster brush borders, and pepsinogen activity. Ng = nanogram (10^{-9} g).

Brush borders isolated from the distal half of hamster intestine were preincubated in 4.0 ml of KRB with or without the addition of hamster gastric juice. After incubation for 60 minutes at 37° C the brush borders were washed 3 times in cold KRB and transferred to a second flask containing either free B_{12}-^{57}Co or B_{12}-^{57}Co bound to hamster gastric juice. After a second incubation for 60 minutes at 37° C, tissue uptake of radioactivity was determined. Preincubation of brush borders with IF significantly ($p < 0.01$) increased the subsequent uptake of free B_{12}-^{57}Co and significantly ($p < 0.01$) inhibited subsequent uptake of B_{12}-^{57}Co bound to IF.

TABLE II

Effect of preincubation of brush borders with IF on subsequent tissue uptake of B_{12}-^{57}Co

Preincubation	Final incubation	B_{12}-^{57}Co uptake*
		pg/mg tissue N
NaCl (control)	B_{12}-^{57}Co	123 ± 15
IF*	B_{12}-^{57}Co	227 ± 18
NaCl (control)	IF†-B_{12}-^{57}Co	1,263 ± 221
IF*	IF†-B_{12}-^{57}Co	692 ± 154

* "IF" = 0.1 ml neutralized hamster gastric juice capable of binding 2.0 ng of cyanocobalamin.
† Mean ± standard deviation of 4 experiments.

Effect of incubation conditions on IF-mediated uptake of B_{12}-^{57}Co by distal brush borders (Table III). Removal of glucose from the incubation medium did not alter uptake of IF-bound B_{12}-^{57}Co by brush borders isolated from the distal hamster intestine. On the other hand, uptake was markedly reduced when brush borders were incubated in KRB that contained no calcium or magnesium ions. IF-mediated attachment of B_{12}-^{57}Co to brush borders was not altered whether incubations were carried out in 95% O_2, room air, or nitrogen.

TABLE III

Effect of incubation conditions on IF-mediated uptake of B_{12}-^{57}Co by brush borders

Medium* containing						
KRB	Glucose	Ca^{++}	Mg^{++}	Atmosphere	Temperature	B_{12}-^{57}Co uptake†
					° C	*pg/mg tissue N*
Changes in medium						
+	+	+	+	95% O_2	37	751 ± 52
+	0	+	+	95% O_2	37	754 ± 46
+	+	0	0	95% O_2	37	253 ± 38
Changes in atmosphere						
+	+	+	+	95% O_2	37	751 ± 52
+	+	+	+	Air	37	783 ± 61
+	+	+	+	N_2	37	810 ± 58
Changes in temperature						
+	+	+	+	Air	7	738 ± 43
+	+	+	+	Air	11	845 ± 37
+	+	+	+	Air	16	819 ± 29
+	+	+	+	Air	25	806 ± 46
+	+	+	+	Air	37	783 ± 61

* To each flask were added 2 ng of B_{12}-^{57}Co and 0.1 ml of a pool of neutralized hamster gastric juice containing a sufficient quantity of IF to bind all the B_{12}-^{57}Co present. All incubations carried out for 60 minutes. KRB = Krelis-Ringer buffer.

Similarly the extent of B_{12}-^{57}Co uptake was not strikingly different over a range of incubation temperatures from 7° C to 37° C.

Effect of supernate obtained from distal intestinal mucosal homogenates on uptake of IF-bound B_{12}-^{57}Co by proximal brush borders. Mucosal scrapings from either the proximal or distal half of hamster intestine were homogenized in 5 mM EDTA and centrifuged at 4° C and 28,500 *g* for 30 minutes in a Sorvall RC-2 high speed centrifuge. To saturate the EDTA, we added 1 ml of 2 M $CaCl_2$ to 40 ml of the resulting clear supernate. The supernate was then preincubated at room temperature for 60 minutes with 2 ml of brush borders isolated from the proximal half of the intestine.

As shown in Figure 7, IF regularly inhibited B_{12}-^{57}Co uptake by brush borders isolated from proximal hamster intestine. When these proximal brush borders were preincubated with supernate obtained after EDTA rupture of proximal intestinal mucosal scrapings (proximal extract), B_{12}-^{57}Co uptake was again inhibited by IF. However, IF consistently enhanced B_{12}-^{57}Co uptake by proximal brush borders that had been preincubated with supernatant fluid obtained after homogenization of distal mucosal scrapings (distal extract). In contrast to the results observed with proximal brush borders, IF did not enhance B_{12}-^{57}Co uptake by

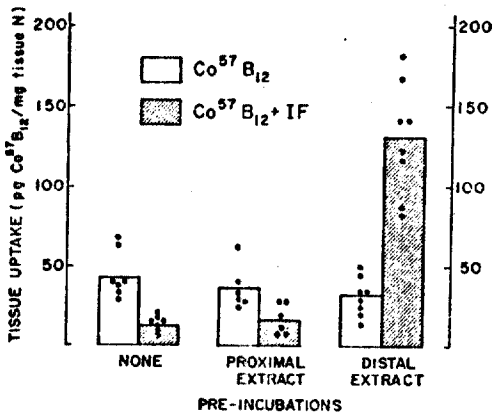

FIG. 7. EFFECT OF PREINCUBATION WITH "EXTRACTS" OF HAMSTER INTESTINE ON IF-MEDIATED UPTAKE OF B_{12}-^{57}Co BY PROXIMAL BRUSH BORDERS. Bars represent mean values and dots indicate results of individual experiments. The "extracts" consisted of supernatant fluid obtained after mucosal scrapings of either proximal or distal hamster intestine had been ruptured with 5 mM EDTA and centrifuged at 28,500 *g* for 30 minutes.

TABLE IV

Effect of preincubation with "distal supernate" on subsequent uptake of B_{12}-^{57}Co by proximal brush borders

Distal supernate	Uptake of B_{12}-^{57}Co*	
	Free B_{12}-^{57}Co	B_{12}-^{57}Co + IF
	pg/mg tissue N	
Untreated	38 ± 12	139 ± 24
Dialyzed	27 ± 14	165 ± 41
Heated 60° C for 10 minutes	34 ± 12	10 ± 6
Centrifuged 34,880 *g*	32 ± 8	144 ± 14
Centrifuged 44,146 *g*	27 ± 9	131 ± 18
Centrifuged 54,500 *g*	33 ± 10	34 ± 12
Centrifuged 105,536 *g*	26 ± 4	21 ± 7
Resuspended pellet†	29 ± 11	137 ± 38

* Mean of 3 to 8 experiments ± standard deviation.
† Pellet obtained after "distal supernate" that had been centrifuged at 54,500 *g* for 30 minutes was resuspended in 40 ml of KRB.

other particles including protein-coated charcoal, calcium phosphate, and homogenates of hamster liver and kidney that had been preincubated with distal supernate.

Proximal brush borders were also preincubated with distal supernates that had previously been dialyzed, heated, or centrifuged at higher speeds (Table IV). Exhaustive dialysis of distal supernate for 48 hours at 6° C against 0.15 M NaCl did not destroy its effect on proximal brush borders. When the distal supernate was heated to 60° C for 10 minutes, however, subsequent uptake of B_{12}-^{57}Co by proximal brush borders was no longer enhanced by IF. A similar loss of effect was consistently observed when distal supernates were centrifuged in the Spinco ultracentrifuge at 54,500 *g* or 105,000 *g* for 30 minutes.

Distal as well as proximal supernates were centrifuged at 54,500 *g* for 30 minutes, and the pellets obtained were resuspended in KRB. Proximal brush borders preincubated with resuspended distal but not proximal pellets again demonstrated IF enhancement of B_{12}-^{57}Co uptake. When the resuspended pellets obtained from distal supernates were incubated with B_{12}-^{57}Co and recentrifuged, it was observed that IF produced a threefold increase in the uptake of B_{12}-^{57}Co by the pellet itself. On the other hand, IF did not enhance B_{12}-^{57}Co uptake by pellets obtained after high speed centrifugation of proximal supernates.

Pellets obtained after centrifugation of distal supernates were fixed and embedded in the same manner as pellets of microvillous membranes.

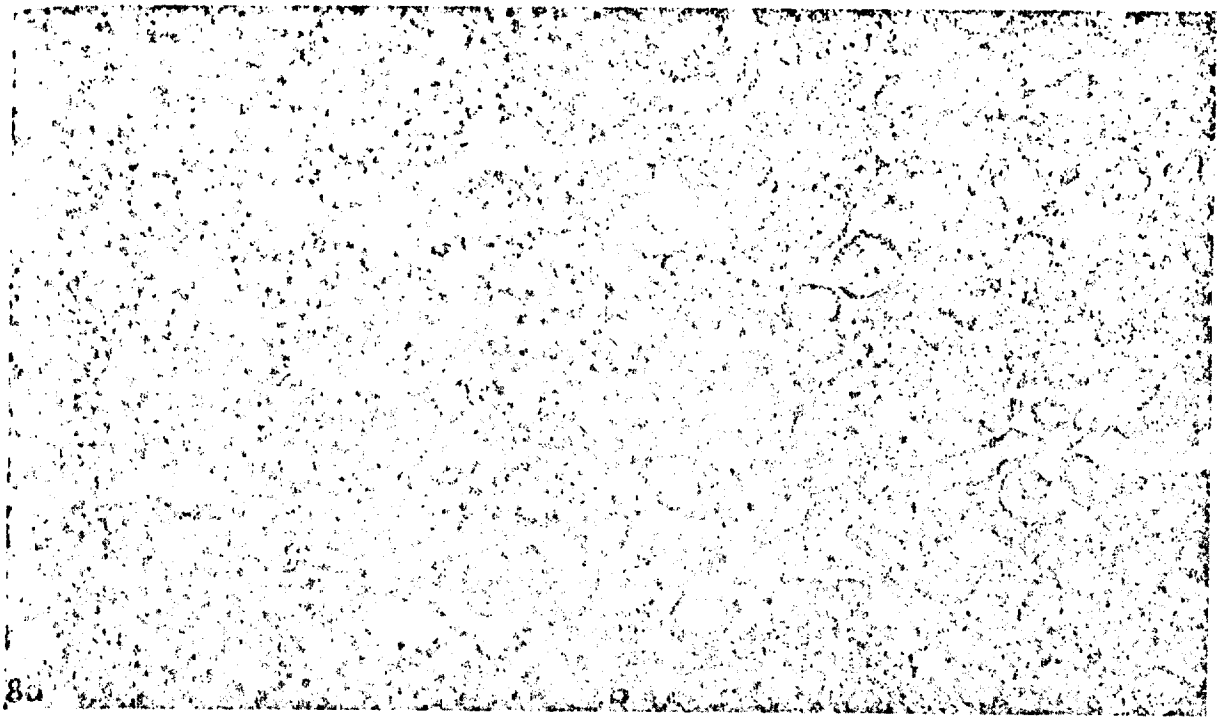

FIG. 8. ELECTRON MICROGRAPHS OF A PELLET OBTAINED AFTER CENTRIFUGATION AT 105,000 G OF SUPERNATE RECOVERED AFTER RUPTURE OF DISTAL INTESTINAL MUCOSAL SCRAPINGS. a) The upper three-fourths of the pellet consists almost exclusively of unattached ribosomes. b) The extreme base contains many membranous fragments in addition to some ribosomes. × 50,000.

This allowed systematic top-to-bottom examination with the electron microscope. The upper three-fourths of the pellet consisted almost entirely of particles of approximately 200 A in diameter, which were identified as unattached ribosomes (Figure 8a). The lower fourth of the pellet contained increasing quantities of membranous fragments in addition to many ribosomes. Membranous elements were most abundant at the extreme base of the pellet (Figure 8b). Exact quantitation of the percentage of ribosomes versus membranes in the pellet was not attempted, but it was estimated that approximately 85% of the pellet was composed of ribosomes, whereas 15% consisted of membranes.

The presence of ribosomes in the pellet was supported by studies with ribonuclease A [2] (recrystallized 3 times). Incubation of pellets for 30 minutes at 37° C with increasing concentrations of ribonuclease A resulted in release of increasing quantities of nucleotides as determined by ultraviolet spectrophotometry (20). Treatment of pel-

[2] Sigma Chemical Co., St. Louis, Mo.

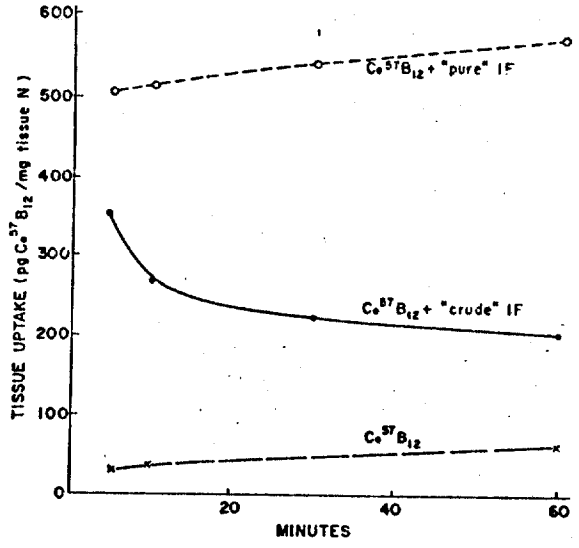

FIG. 9. EFFECT OF DURATION OF INCUBATION ON IF-MEDIATED UPTAKE OF B_{12}-^{57}Co BY DISTAL BRUSH BORDERS. Each dot represents mean of 2 or 3 experiments. "Crude" IF consisted of saline extract of hamster gastric mucosa. "Purified" IF consisted of peak B_{12} binding fraction obtained after gel filtration of crude extract. In each experiment sufficient IF to bind 2 ng of vitamin B_{12} was added to incubation flasks. Uptake of B_{12}-^{57}Co in absence of IF is shown for comparison.

lets with sufficient enzyme to produce maximal nucleotide release, however, did not impair IF-mediated uptake by these pellets.

Lysis of brush borders by hamster gastric mucosal extract and neutralized hamster gastric juice. When a crude saline extract of hamster gastric mucosa was used as the source of IF, uptake of B_{12}-^{57}Co by distal brush borders decreased rather than increased as the duration of incubation was prolonged (Figure 9). No such decrease was observed, however, when this same gastric mucosal extract was used after partial purification by gel filtration on Sephadex G-200. Furthermore, as shown in Figure 10, uptake of ^{57}Co by distal brush borders increased with increasing purity of the IF preparation used. Purity of IF preparations was assessed on the basis of vitamin B_{12} binding capacity per milligram of nitrogen, and the quantity of each preparation added to the incubation medium was just sufficient to bind 2 ng of B_{12}-^{57}Co.

Since these results suggested that a factor which inhibited tissue uptake of B_{12}-^{57}Co might be present in crude sources of IF, we examined the ef-

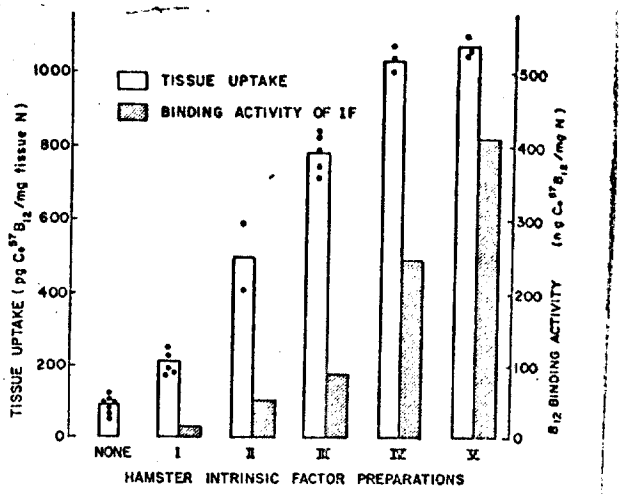

FIG. 10. EFFECT OF PARTIAL PURIFICATION OF HAMSTER IF PREPARATIONS ON UPTAKE OF B_{12}-^{57}Co UPTAKE BY DISTAL BRUSH BORDERS. In each uptake experiment the quantity of IF added was just sufficient to bind 2 ng of B_{12}-^{57}Co. Hatched bars indicate relative purity of IF preparations in terms of B_{12} binding capacity per milligram of nitrogen. Hamster IF preparations included saline extract of gastric mucosa (I), *in vivo* neutralized gastric juice (III), and various fractions (II, IV, V) obtained after gel filtration of crude sources.

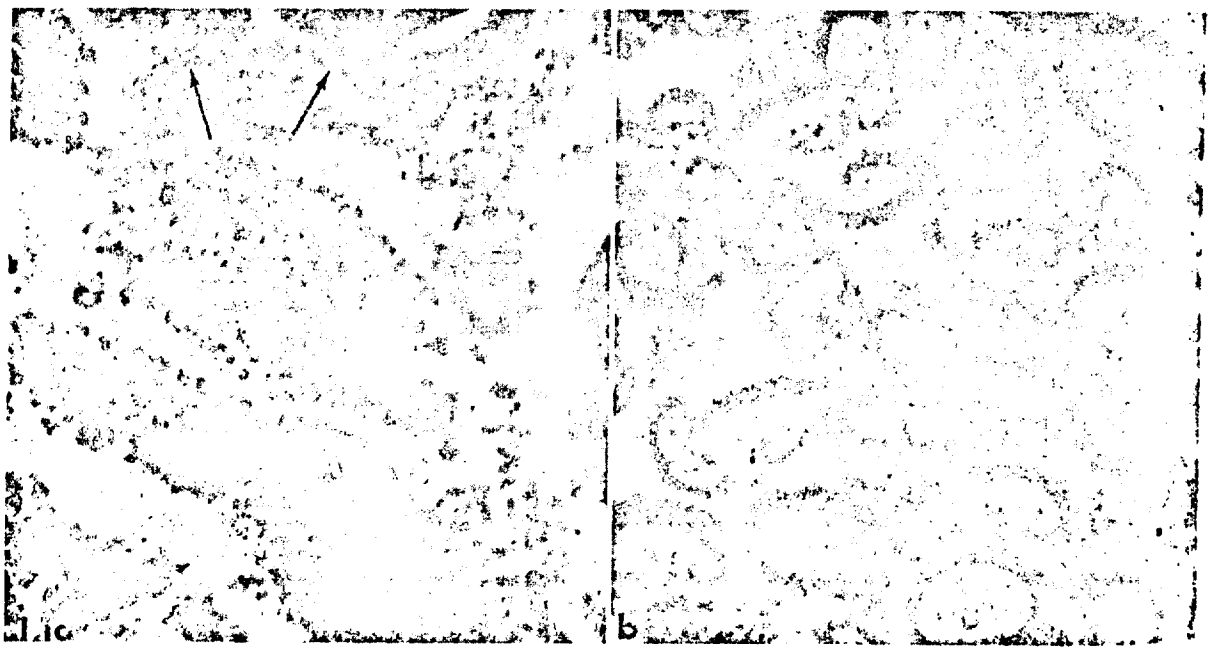

FIG. 11. LIGHT MICROGRAPHS OF PELLETS OF BRUSH BORDERS INCUBATED WITH CRUDE AND PARTIALLY PURIFIED HAMSTER IF. Incubations carried out at 37° C for 10 minutes with saline extract of gastric mucosa (crude IF) and saline extract after gel filtration on Sephadex G-200 (partially purified IF). a) After incubation with crude IF, large amounts of amorphous material (A) are seen. Only a few, pale-staining, degenerating brush borders (arrows) can be identified. Epon embedment, toluidine blue, × 2,000. b) After incubation with partially purified IF, the brush borders remain well preserved and appear comparable to pellets of unincubated brush borders (compare to Figure 1). Epon embedment, toluidine blue, × 2,000.

fect of crude and partially purified IF on the morphologic appearance of brush borders. Figure 11a is a light micrograph of a representative field of a pellet of brush borders incubated with a crude saline extract of hamster gastric mucosa for 10 minutes at 37° C. Large amounts of amorphous material, never seen in control pellets, are present. A few faintly stained degenerating but still recognizable brush borders can be identified. Figure 11b is a light micrograph of a representative field of a pellet of brush borders that had been incubated with a partially purified saline extract of hamster gastric mucosa for 10 minutes at 37° C. In contrast to those in Figure 11a, the brush borders are still intact after incubation and appear quite comparable to those seen in unincubated preparations (compare with Figure 1). Similar but less marked effects were observed with *in vivo* neutralized hamster gastric juice. Further support for the destructive effect of crude sources of IF was derived from the observation that addition of increasing quantities of gastric mucosal extract resulted in increased release of tissue nitrogen into the incubation medium.

Discussion

Intrinsic factor consistently enhanced uptake of ^{57}Co-labeled cyanocobalamin by brush borders and microvillous membranes isolated from the villous cells of the distal half of hamster small intestine. This observation, together with the fact that uptake of IF-bound B_{12}-^{57}Co by microvillous membranes was tenfold greater per milligram of tissue nitrogen than was uptake by homogenates of whole intestinal mucosa, suggests localization of IF action *in vitro* to the apical surface of the intestinal cell. The highly developed mucopolysaccharide surface coat that is applied to the outer leaflet of the microvillous unit membrane was retained in these preparations of brush borders and microvillous membranes (Figures 2 and 3). There is now good evidence (21) that this surface coat is an integral part of the apical plasma membrane rather than simply adsorbed extraneous material. The present studies, however, do not demonstrate whether IF promoted vitamin B_{12} uptake by the unit membrane itself or by its surface coat.

Several observations establish the specificity of the observed IF-mediated attachment of B_{12}-^{57}Co to the apical cell membrane isolated from the villous absorptive cell surface and suggest that the process is of physiological significance. Enhancement of tissue uptake of B_{12}-^{57}Co by IF was observed only with brush borders and microvillous membranes isolated from the distal half of the intestine. An effect of IF on vitamin B_{12} uptake by distal but not proximal intestine has previously been observed with everted sacs (22) and homogenates (23) of intestine. In addition, IF-mediated absorption of the vitamin *in vivo* appears to occur at the distal rather than the proximal intestine of man (24). Only IF from hamsters, rats, and rabbits promoted B_{12}-^{57}Co uptake by hamster brush borders; human, hog, and dog IF preparations were without effect. Except that hog IF was slightly active, this same species specificity was observed with everted sacs of hamster intestine (25). Finally, a direct relation was demonstrated between enhancement of tissue uptake of B_{12}-^{57}Co and the vitamin B_{12} binding activity of hamster gastric juice or hamster gastric mucosal extract after fractionation of these sources of IF by gel filtration (Figure 5) or ion exchange chromatography (Figure 6).

The latter observation is consistent with previous studies which suggest that binding of vitamin B_{12} in a macromolecular complex plays an important role in IF-mediated absorption of the vitamin *in vivo* (3). When both free and IF-bound vitamin B_{12} are present in man (26, 27) and in rats (27), the IF-bound vitamin is preferentially absorbed. When human gastric juice is subjected to ion exchange chromatography with Amberlite CG-50, two peaks of vitamin B_{12} binding activity are obtained, but only the second and larger peak is able to promote vitamin B_{12} absorption in patients lacking IF (17). When hamster gastric mucosal extract was similarly fractionated, only the second peak significantly enhanced B_{12}-^{57}Co uptake by brush borders isolated from hamster intestine. It should be emphasized, however, that not all substances that bind vitamin B_{12} promote its absorption (3), and in the present study non-IF binders of vitamin B_{12} in serum, saliva, and tissue extracts failed to stimulate B_{12}-^{57}Co uptake by brush borders (Table I).

On the basis of studies of IF action *in vitro*, Herbert (5, 28) postulated a specific receptor for the IF-vitamin B_{12} complex located on the surface

of intestinal cells and suggested that the IF molecule contains two active sites, one which binds vitamin B_{12} in a macromolecular complex and one which attaches to the intestinal receptor. The present studies with brush borders and microvillous membranes support the concept that this postulated receptor is located on the apical cell membrane. In addition, sequential incubation of brush borders provided results consistent with those previously described in rat liver slices (28) and everted intestinal sacs (5). In our studies, brush borders, preincubated with IF alone, subsequently took up more free B_{12}-^{57}Co than did control preparations, whereas uptake of IF-bound B_{12}-^{57}Co was inhibited when brush borders were preincubated with IF (Table II). These observations suggest that *1*) IF in the absence of vitamin B_{12} is able to attach to the postulated intestinal receptor, *2*) free B_{12}-^{57}Co subsequently becomes bound to receptor-attached IF, and *3*) uptake of IF-bound B_{12}-^{57}Co is reduced after preincubation with IF alone because receptor sites have been "covered." Since preincubation of IF with brush borders produced only partial enhancement of free B_{12}-^{57}Co uptake and only partial inhibition of uptake of IF-bound B_{12}-^{57}Co, it is possible that, under these experimental conditions, the intestinal receptor more readily accepts the IF–B_{12}-^{57}Co complex than IF alone. It should be pointed out, however, that in some experiments with everted intestinal sacs (4, 22), preincubation of tissue with IF failed to stimulate subsequent uptake of vitamin B_{12}.

IF enhancement of B_{12}-^{57}Co uptake was not diminished when brush borders were incubated in the absence of glucose or oxygen and was not significantly altered by changes in incubation temperature from 7° C to 37° C (Table III). This suggests that the observed attachment of IF-bound B_{12}-^{57}Co to brush borders, although specific, resulted from adsorption and did not depend upon an energy-requiring enzymatic process. The observation that prolonged incubation did not significantly increase uptake of IF-bound B_{12}-^{57}Co (Figure 9) supports this view. A role for divalent cations in the attachment process is suggested by the decreased uptake of IF-bound B_{12} in incubation mixtures devoid of calcium and magnesium ions (Table III). These results agree with the observations of Sullivan, Herbert, and Castle (7), who showed that IF enhancement of vitamin B_{12} uptake by guinea pig intestinal mucosal homogenates occurs in the absence of glucose or oxygen, is not markedly affected by temperature changes or time, and requires the presence of divalent cations. Everted sacs of intestine may constitute a more complex system, however, since IF-mediated uptake of vitamin B_{12} by this tissue preparation is markedly inhibited by anaerobic conditions, absence of glucose, and low incubation temperatures (22). It is possible that uptake by the relatively intact intestinal mucosa of everted sacs may include an energy-dependent step that is lost when mucosal cells are homogenized. In any case, direct demonstration of an IF effect upon intestinal brush borders and microvillous membranes makes feasible isolated examination of the initial attachment phase of vitamin B_{12} absorption as well as more precise localization and characterization of an intestinal receptor for the IF-vitamin B_{12} complex.

The possibility that other subcellular fractions may also affect intestinal brush border uptake of IF-bound B_{12}-^{57}Co was raised by experiments in which proximal brush borders were preincubated with the supernatant fluid ("distal supernate") obtained from homogenization of distal intestinal mucosa (Figure 7). After preincubation with this distal supernate, proximal brush borders appeared to behave like distal brush borders in that IF clearly enhanced uptake of B_{12}-^{57}Co. Preincubation with supernatant fluid obtained after homogenization of proximal intestinal mucosa did not produce this effect.

Since the supernatant fluid was obtained after centrifugation at 28,500 *g* and appeared to be perfectly clear, our initial interpretation of this experiment (1) was that a soluble "receptor" for the IF-vitamin B_{12} complex had been transferred from the distal mucosa to proximal brush borders. This view is no longer tenable, however, since it was subsequently demonstrated that centrifugation at higher speeds (greater than 54,500 *g*) yielded a small pellet, and that the resultant supernate then has no effect on proximal brush borders. Since, in addition, the resuspended pellet obtained after high speed centrifugation of distal supernate was capable of causing subsequent IF enhance-

of B_{12}-^{57}Co uptake by proximal brush borders, it would appear that the active factor in distal supernate is present in a particulate form. IF also promoted enhanced uptake of B_{12}-^{57}Co by the pellet itself.

This pellet was found to consist largely of unattached ribosomes together with a relatively small quantity of membranous fragments (Figure 8). Further studies are required to determine the exact source of the membranous fragments, but it seems likely that some are derived from intracytoplasmic membranous organelles, whereas others represent fragments of plasma membrane. The mechanism by which these subcellular particles result in IF enhancement of B_{12}-^{57}Co uptake by proximal brush borders is under investigation. Whether the ribosomes or the membranes are responsible and how they mediate IF enhancement of B_{12} uptake remain to be determined.

Incubation of brush borders with crude hamster IF preparations resulted in marked destruction of normal architecture (Figure 11). Partial purification of these IF preparations by gel filtration eliminated this destructive effect (Figure 11b). Brush border destruction was accompanied by release of tissue nitrogen into the incubation medium, suggesting that a proteolytic enzyme was responsible. The effect cannot be attributed to peptic activity since incubations were carried out at pH 7.4. The observations that *1*) prolonged incubation of brush borders with crude hamster IF resulted in decreased rather than increased uptake of B_{12}-^{57}Co and, *2*) partially purified IF preparations were more effective than crude preparations in promoting B_{12}-^{57}Co uptake suggest that the destructive action of crude IF sources interfered with uptake of B_{12}-^{57}Co by brush borders. Studies with guinea pig intestinal mucosal homogenates (*7*, 23) have shown that tissue uptake of vitamin B_{12} is impaired in the presence of excessive quantities of neutralized human gastric juice. This inhibitory effect of crude IF excess may be explained in part by the presence of a factor that lyses brush borders *in vitro*. Further studies are required, however, to determine whether the inhibition caused by excessive quantities of crude IF may also be due in part to covering of intestinal receptor sites by IF molecules to which no vitamin B_{12} is bound (5).

Acknowledgments

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PHYSIOLOGIC ASPECTS OF AGING. II. RADIOCYANOCOBALAMIN ABSORPTION AND PROTEIN DIGESTION BY THE RAT

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As part of a study of the relationship between aging and the absorption and metabolism of various nutrients by rats, the absorption of radioactive cyanocobalamin and the digestion of protein have been investigated. Previous work has indicated that the efficiency of absorption of radiothiamine by the rat does not change appreciably up to about 20 months of age, beyond which a substantial reduction occurs (5). The present experiments suggest that a reduction in the efficiency of cyanocobalamin absorption also occurs in aged rats and that the digestion of protein is unimpaired.

MATERIAL AND METHODS

Male albino rats of the Sprague-Dawley strain were maintained on a commercial stock diet (ground Purina Laboratory Chow) with a supplement of 2500 I.U. of vitamin A per month. A total of 31 animals, ranging in age from 2.5 to 23 months, was selected from the pool of animals, fasted for 24 hours, and placed in a urinofecal separator (6). A dose of 0.1 mcg. of Co^{60}-cyanocobalamin in 0.25 ml. aqueous solution (approximately 44,000 disintegrations per minute) was administered by stomach tube under light ether anesthesia. This quantity of the vitamin represents an amount approximately equivalent to the daily requirement of the growing rat. The feces were collected for a 48-hour period, during which *ad libitum* access to the stock diet was given.

The feces were dried for 12 hours at 105 C. and mixed thoroughly by pulverizing to a fine powder. A sample of this material was spread uniformly in a cupped planchet, covered with a layer of aluminum foil to eliminate the effect of beta particle emissions, and counted in a Tracerlab Q-gas windowless gas-flow counter connected to a Nuclear scaler. A self-absorption curve was prepared by adding a solution of Co^{60}-cyanocobalamin to dried, ground feces and, after drying and remixing, counting suitable amounts of the radioactive material. All sample counts were corrected for self-absorption and decay. The radioactivity excreted in the feces of the rat after an oral dose of Co^{60}-cyanocobalamin has been shown to represent a mixture of the unabsorbed vitamin and inorganic Co^{60} released by bacterial decomposition (1). Injection of Co^{60}-cyanocobalamin leads to an insignficant excretion of radioactivity in the feces.

The digestibility of the protein of the stock diet was determined for rats of different ages by feeding a standard amount of an air-dried reference sample. After a 12-hour fast, each animal was given 10 Gm. of pulverized diet which had been mixed with a trace quantity of ferric oxide. When the marked sample had been consumed, free access was given to the unmarked stock diet. All of the marked fecal pellets were collected during the subsequent period of 2 or 3 days. The nitrogen content of the marked feces and the test diet was determined by the Kjeldahl method, and protein was calculated on the basis of 16% nitrogen. The apparent digestibility coefficient (A.D.C.) was calculated according to the fraction of dietary nitrogen apparently absorbed.

RESULTS AND DISCUSSION

The extent to which an oral dose of 0.1 mcg. of cyanocobalamin is absorbed by rats of different ages is shown in figure 1. Statistical treatment indicated that two trends are represented by the data, namely, an increase in absorption which accompanied maturation and a decrease which accompanied senility. Absorption of the vitamin was significantly greater in the 8-months-old group than in the 2.5-months-old growing animals ($P = <.01$). This difference may be primarily a function of body weight, which ranged from 175-200 Gm. for the youngest group in contrast to 400-500 Gm. for most of the adult animals. A significant correlation between body weight and cyanocoba-

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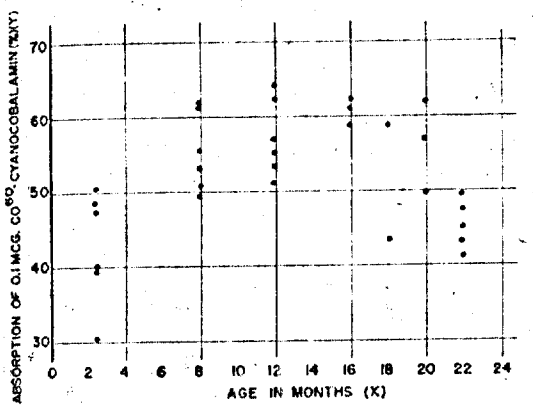

Fig. 1. Intestinal absorption of Co^{60}-cyanocobalamin by rats of various ages.

lamin absorption was found ($r = 0.62$; $P = <.01$), which is largely attributable to the lower uptake by the growing animals.

A regression analysis of the data relating to the rats from 12 to 22 months old revealed a significant reduction in absorption over this span ($P = <.01$). However, it may be seen in figure 1 that no consistent decrease is evident until 22 months of age, when an average absorption of 45% was observed compared with 58% at 12 months.

The digestibility of dietary protein by rats of various ages is illustrated in figure 2. Considerable variability within age groups is evident and no differences associable with age were discernible, the regression of age on protein digestibility being statistically insignificant ($P = >0.2$).

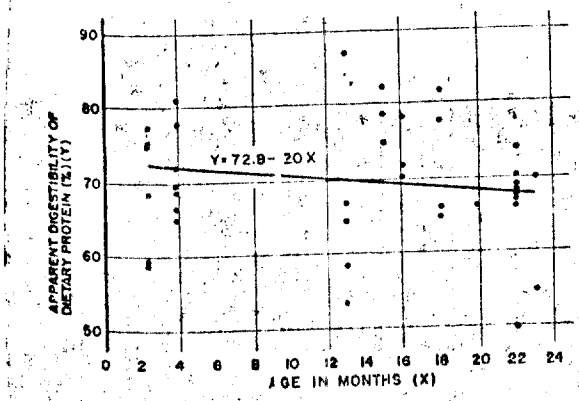

Fig. 2. Apparent digestibility of protein by rats of various ages.

The A.D.C. of protein determined by the foregoing procedure represents a composite estimate of the hydrolysis of dietary protein in the gastrointestinal tract and of the absorption of the end products of hydrolysis. It does not take into account that fraction of fecal nitrogen which is of body origin, and therefore underestimates the true digestibility of the dietary protein. Since the amount of metabolic fecal nitrogen is primarily a reflection of the dry matter content of the diet, which was equalized in this experiment, the standardized procedure used provides a valid basis for comparing animals of different ages.

The results on cyanocobalamin absorption suggest that a reduction in the absorption of this vitamin occurs at an age comparable to that previously observed for thiamine, although in the latter case the decline was of somewhat greater magnitude. These findings are of interest in connection with those of Chow and coworkers (4), who reported that the vitamin B_{12} concentration in the plasma of rats declines steadily with advancing age from 0.5 months to senility. To the extent that the efficiency of absorption affects plasma concentration, the blood level would tend to be lower, according to the present experiment, in immature and aged animals, with little change during other periods. The voluntary food intake of the growing rat in relation to body weight is known to decrease according to a quadratic function as the animal matures (9). Thus the absolute intake of vitamin B_{12} per unit of plasma volume, when fed in a diet of constant composition, is approximately twice as great for a 50 Gm. rat as for a 250 Gm. rat. This difference between the intake of young and mature animals is considerably greater than the apparent disparity in absorption, and may account for the higher plasma concentration found in young rats (4). Among adult animals the average decline of 17% in plasma concentration between 13 and 23 months observed by Chow and associates compares with the average decrease of 18% in absorption, calculated by regression analysis, between 12 and 22 months in the present experiment. This similarity implies that the decrease in plasma cyanocobalamin level may be a reflection of decreased uptake by the intestine.

These observations on the rat are in general conformity with those reported for the adult

human by Glass and associates (8), who observed no general regression between hepatic uptake of Co^{60}-cyanocobalamin and age but noted a higher incidence of individuals in the age group between 50 and 80 years who exhibited a low uptake. Evidence that the serum concentration of vitamin B_{12} bears an inverse relationship to age in several selected human populations has been presented by Boger and associates (2), Chow and coworkers (4), and Gaffney and associates (7).

The absence of a significant trend in protein digestion associated with age in the rat is analogous to the failure of Sharp and coworkers (11) to find a correlation between the digestion of N^{15}-labeled yeast protein and aging in human subjects. Chinn, Lavik, and Cameron (3) have reported similar findings using I^{131}-labeled protein. The experiments of Meyer, Spier, and Neuwelt (10), however, indicated that the fasting secretion of pepsin and trypsin is impaired in aged humans.

SUMMARY

The absorption of a standard oral dose of 0.1 mcg. of Co^{60}-cyanocobalamin, determined from the fecal excretion of Co^{60}, was studied as a function of age in the rat. In general, adult rats absorbed a larger fraction of the dose than immature animals except in the case of the oldest age group (22 months), which exhibited a decrease in uptake. The digestibility of the protein in a reference sample of stock diet was determined for rats ranging from 2.5 to 23 months of age, with no agewise differences being found.

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Causes of the Increased Requirement for Vitamin B_{12} in Rats Subsisting on an Unheated Soybean Flour Diet [1]

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ABSTRACT Possible causes of the increased requirement for vitamin B_{12} by rats fed a diet of unheated soybean flour were investigated. Decreased intestinal synthesis or increased destruction, as well as decreased absorption from the intestine were excluded. However, the vitamin B_{12} formed by the intestinal flora was less available for absorption when synthesized by rats subsisting on unheated soybean flour. Furthermore, turnover of vitamin B_{12} in these rats, as estimated by the rate of disappearance of injected ^{57}Co-labeled vitamin B_{12} from kidneys, liver and spleen, was slightly accelerated. J. Nutr. *100:* 1377–1382, 1970.

It has been reported (1, 2) that feeding unheated soybean flour (SBF) increases the requirement for vitamin B_{12} and enhances metabolic disorders characteristic of vitamin B_{12} deficiency. The levels of vitamin B_{12} in the liver, kidney and blood serum were lower in these animals than in controls receiving heated SBF. Following a load with propionate or histidine, urinary excretion of methylmalonic acid (MMA) and formiminoglutamic acid, respectively, was increased. Addition of vitamin B_{12} to the diet improved weight gain and decreased MMA excretion. When vitamin B_{12} was added to a diet of heated SBF, weight gain was not increased and MMA excretion was only slightly reduced. These results gave strong evidence of an increased requirement for vitamin B_{12} by rats fed unheated SBF.

The present investigation was undertaken in order to determine the possible causes of this increased requirement. Since the diets were devoid of vitamin B_{12}, the only possible source of the vitamin was that formed by the intestinal flora. Feeding unheated SBF may adversely affect this supply by decreasing intestinal synthesis or increasing destruction. Unheated SBF contains a goitrogenic factor (3), and decreased thyroid activity has been shown to impair absorption of vitamin B_{12} (4). Therefore, an increased vitamin B_{12} requirement may also be due to an impaired absorption of the vitamin. Another possibility is a decreased availability of fecal vitamin B_{12}. The vitamin B_{12} formed by intestinal microorganisms is not absorbed in the cecum and colon, the main sites of synthesis, but only during a "second passage" in rats practicing coprophagy (5). It is thus possible that the vitamin B_{12} formed in rats fed unheated SBF is less available to the organisms. Lastly, differences in turnover and utilization of absorbed vitamin B_{12} have been considered.

METHODS

Animals and diets. Young male rats weighing 30 to 40 g, of a local strain derived from Wistar rats, were used in these experiments. The diets provided 18% protein derived from low fat soybean flour, either unheated or commercially heat-processed. The SBF was supplemented with vegetable oil to give 5% lipids in the ration, 4% salts (USP XIV) and with cornstarch to make up to 100%. All diets were enriched with the conventional vitamins,[2] vitamin B_{12} being omitted.

Test meals. Test meals consisted of a 25% (w/v) aqueous suspension of SBF, either heated or unheated, of which 2 ml

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[2] The vitamin mix consisted of: (in mg/kg ration) thiamin, 2.0; riboflavin, 3.0; pyridoxine, 1.6; calcium pantothenate, 12.0; niacin, 30.0; choline chloride, 1,000; retinol propionate, 2.4; and cholecalciferol, 0.02.

per 100 g body weight were administered by stomach tube.

Prevention of coprophagy and collection of feces. A plastic container with the bottom removed and a hole bored in the cover was fastened onto the tail of the rat so as to completely cover the anus (fig. 1). Plastic scintillation vials (Packard) were suitable. A strip of adhesive tape was fixed on the tail distally to the hole and a second strip was wound around it to hold the vessel in place. The second strip was removed when the container was changed, whereas the first strip remained in place during the whole experimental period protecting the tail skin which would be irritated by frequent removing of the tape. When necessary, this device was improved by winding adhesive tape around the vessel to prevent it being gnawed at by the rat, and one strip at its proximal end to prevent irritation of the skin by the sharp edge of the vessel. This device which is a modification of that developed by Barnes et al. (6) completely prevented coprophagy and allowed for feces collection. It was used for estimation of fecal vitamin B_{12}, for prevention of coprophagy in absorption studies and for collection of feces of donor rats in availability studies.

Measurement of radioactivity. ^{57}Co-labeled vitamin B_{12} [3] was administered by stomach tube or intraperitoneally. Five nanograms with a specific activity of 5.76 μCi/μg were used, unless otherwise stated. Radioactivity of feces, blood and organs was measured in a gamma ray spectrometer.[4]

In vivo absorption of vitamin B_{12}. Absorption was studied with labeled vitamin B_{12}. In the first experiment of this series two groups, each consisting of six rats, were maintained for 7 weeks on a diet with heated or unheated SBF. Following a fast of 15 hours, 7.5 ng of labeled vitamin B_{12} with a specific activity of 60 μCi/μg were administered by stomach tube. During the following 2 hours, food was withheld. Feces were collected daily for 7 days with the device described, dried overnight at 120°, and radioactivity was measured. The amount of radioactivity not excreted was considered to be absorbed.

In order to avoid, as far as possible, faulty conclusions through ignoring reexcretion of absorbed vitamin B_{12} via the bile into the gastrointestinal tract (7), another experiment was conducted on 14 rats maintained as described above. After a fast of 15 hours they were dosed with labeled vitamin B_{12} by stomach tube. Coprophagy was not prevented in any of these rats prior to the fast. After specified time intervals one pair of each group was killed.

[3] Radiochemical Centre, Amersham, England.
[4] Packard Auto Gamma Spectrometer, Model 3002, Packard Instrument Co., Inc., Downers Grove, Ill.

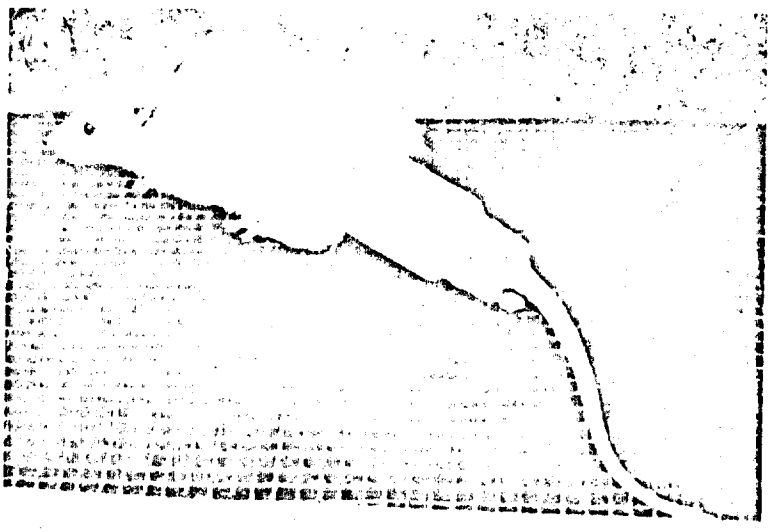

Fig. 1 Rat with device for prevention of coprophagy and feces collection.

Their small intestines were removed, thoroughly washed with saline and the radioactivity of the wall of the small intestine, and of a blood sample, was measured. Coprophagy was prevented in rats killed 24 and 48 hours after treatment.

In animals in which absorption was estimated immediately following a test meal of SBF, meals were administered to rats previously fed laboratory chow and fasted for 15 hours before the test meal. Radioactive vitamin B_{12} was then given by stomach tube. The rats were killed after 2 hours and radioactivity of the wall of the small intestine was determined. Coprophagy was not prevented in these rats.

Availability of vitamin B_{12}. Availability of fecal vitamin B_{12} was estimated in rats fed for 7 weeks on heated or unheated SBF diets. During the last 3 weeks, feces of other rats were added to the diet. Feces were collected from a sufficiently large group of donor rats during 1 day, immediately lyophilized, finely milled, and their vitamin B_{12} content was examined. The dried feces were kept in the cold until use. The respective diets were supplemented with feces to give 5 μg vitamin B_{12} per kilogram. Coprophagy was not prevented in the rats fed these diets.

Turnover of vitamin B_{12}. Vitamin B_{12} turnover was estimated in rats which were fed for 4 weeks on heated or unheated SBF diets. In order to eliminate, as far as possible, differences in tissue stores of vitamin B_{12}, 14 rats of each group were loaded with unlabeled vitamin B_{12} by giving three intraperitoneal injections of 5[illegible] μg unlabeled vitamin B_{12} at 2-day intervals. After the third injection, radioactive vitamin B_{12} was injected. One pair of each group was killed after specified time intervals and the radioactivity of kidneys, liver and spleen measured. The radioactivity at these time periods was calculated relative to that of one day, expressed in percentage.

Propionate loading and MMA excretion. The procedures have been described in a previous report (1).

Estimation of vitamin B_{12}. Vitamin B_{12} in feces and cecal content was estimated microbiologically (8). Feces were collected for 24 hours as described and examined on the day of collection.

RESULTS AND DISCUSSION

In the first experiment vitamin B_{12} content of feces and cecal content was examined in rats fed diets containing heated or unheated SBF for a period of 10 weeks. Feces were collected with the device described. The results given in table 1 fail to show any significant difference in vitamin B_{12} content between the two groups. Therefore, the increased vitamin B_{12} requirement by rats fed unheated SBF can hardly be attributed to a decreased intestinal synthesis or an increased destruction.

In further experiments, the absorption of vitamin B_{12} was examined. First, feces were collected daily for 7 days with the device described from rats previously given radioactive vitamin B_{12} by stomach tube, and the radioactivity was measured. In rats fed unheated SBF (U rats), 53.5± 3.2% (mean±SE) of the dose was absorbed, whereas in rats fed heated SBF (H rats) 47.8±3.7% were absorbed. The difference was not statistically significant.

In the next experiment, radioactivity of intestine and blood of rats was examined at different time intervals following a dose of radioactive vitamin B_{12}. In experiments lasting 24 or 48 hours coprophagy was prevented. The results are shown in table 2. During the first 30 minutes following administration of labeled vitamin B_{12} more

TABLE 1

Vitamin B_{12} levels in feces and cecal content of rats fed diets with heated and unheated soybean flour for a period of 10 weeks

Soybean flour in diet	Feces		Cecal content	
	µg/g dry feces	*µg/100 g body weight/day*	*µg/g dry weight*	*µg/100 g body weight*
Heated	1.79±0.29 [1]	1.48±0.14	1.90±0.33	0.66±0.10
Unheated	1.66±0.09	1.92±0.22	1.20±0.24	0.54±0.10

[1] All results are expressed as averages ± SEM of nine rats. There were no significant differences between treatments.

radioactivity was absorbed by the intestinal wall of H rats, whereas later absorption appears to be higher in U rats. A similar trend is discernible in blood. These results indicate that the vitamin B_{12} deficiency developed in U rats is apparently not due to impaired absorption. Absorption was delayed in U rats, possibly because gastrointestinal movements were slower in rats adapted to unheated SBF. A slow rate of food passage through the stomach has been reported for rats fed unheated SBF (9). The higher absorption of radioactive vitamin B_{12} by U rats results possibly from their depletion of this vitamin. In a similar experiment with rats on a not specified and presumably standard diet the intestinal wall showed maximum activity 0.5 to 2 hours after dosing. No radioactivity appeared in the blood during the first hour, and the peak was at 4 hours (10).

Another experiment was conducted in which the labeled vitamin was given immediately after a test meal of SBF. In five rats given a meal of unheated SBF, 31.4± 1.19% of the radioactivity was recovered. The percentage recovered in five control rats receiving a meal of heated SBF was 33.4±0.85. This difference was statistically nonsignificant.

It appears that differences in absorption of vitamin B_{12} fail to play a role in the causation of the deficiency of this vitamin developing in rats fed unheated SBF.

Availability of intestinally formed vitamin B_{12} was studied in five groups consisting of five or six rats which had been fed a diet of either heated or unheated SBF. To determine whether the vitamin B_{12} in feces of U rats is possibly less available for absorption during the second passage by rats practicing coprophagy than when in feces of H rats, the unheated SBF diet was supplemented with feces from either H or U rats, and feces from H rats were added to the heated SBF diet, as indicated in table 3. Coprophagy was not prevented in rats fed these three diets. At the end of

TABLE 2

Radioactivity of intestinal wall and blood of rats fed soybean flour diets and given radioactive vitamin B_{12} by stomach tube

Hr after vitamin administration	Intestinal wall				Blood			
	Heated [1]		Unheated [2]		Heated [1]		Unheated [2]	
	cpm				*cpm/ml*			
½	38,350 [3]	35,307	30,138	30,000	0	0	0	0
1	30,044	32,614	42,712	40,561	7	18	1	1
2	35,167	26,974	33,176	36,234	52	56	72	83
4	8,922	12,491	13,256	12,900	38	45	68	67
8	5,129	3,337	5,028	4,570	33	29	54	51
24	2,711	3,563	3,119	3,971	10	19	17	22
48	2,404	835	3,719	3,298	6	0	17	16

[1] Rats fed heated soybean flour diet.
[2] Rats fed unheated soybean flour diet.
[3] Results are expressed as mean cpm per total intestinal wall and per 1 ml of blood. Each figure refers to one rat.

TABLE 3

Excretion of methylmalonic acid by rats fed heated or unheated soybean flour supplemented with feces of other rats

Soybean flour in diet	Feces added	Excretion of methylmalonic acid with urine	
		mg/24 hr	*mg/24 hr/100 g body wt*
Heated	—	28± 5.5 [1]	10±1.8 [a,2]
Unheated	—	63±12.6	38±5.8 [b]
Heated	Heated [3]	21± 4.8	8±1.8 [a]
Unheated	Unheated [4]	43± 4.3	31±2.4 [b]
Unheated	Heated [3]	25± 3.3	17±2.4 [a]

[1] All results are expressed as averages ± SEM of five or six rats.
[2] Values followed by different letters are significantly different, $P < 0.01$.
[3] Feces of rats fed a heated soybean flour diet.
[4] Feces of rats fed an unheated soybean flour diet.

a 3-week observation period a load of sodium propionate was administered, urine collected for 24 hours and excretion of MMA determined. As expected from our previous work (1), unsupplemented U rats excreted more MMA than H rats. Adding feces of H rats to heated SBF did not increase excretion of MMA. However, addition of feces from H rats to unheated SBF resulted in a significantly lower excretion than addition of feces from U rats ($P < 0.01$). These results support the assumption that fecal vitamin B_{12} of U rats is less available for absorption than that of H rats. No easy explanation offers itself. It is possible that vitamin B_{12} formed by the intestinal flora is bound to substances appearing in the intestine during digestion of unheated SBF.

Lastly, vitamin B_{12} turnover was investigated; coprophagy was not prevented in this experiment. Most of the radioactivity injected was found in the kidneys. Twenty-four hours after injection, 29% of the injected dose was recovered per gram kidney of H rats as against 39% per gram kidney of U rats. Time curves of retained radioactivity are presented in figure 2. On day 3 radioactivity increased in both groups, in U rats more than in H rats. From this day on, radioactivity decreased in both groups and in U rats more than in H rats. On day 27 there was almost no difference in kidney radioactivity between the two groups. In liver about 1% only of the injected radioactivity was found per gram tissue, in both H and U rats, 24 hours following injection. It increased markedly in H rats, whereas in U rats the increase was of a short duration, followed by a marked decrease. In spleen less than 1% of administered radioactivity was recovered per gram tissue after 24 hours, in both H and U rats. Radioactivity decreased considerably, in U rats more than in H rats. It appears that utilization of injected vitamin B_{12} is more rapid in U rats than in H rats. This increased turnover may also contribute to the more rapid depletion of vitamin B_{12} appearing in rats fed unheated SBF. In a similar experiment performed on rabbits, Rosenthal (11) reported that liver, kidneys and spleen accumulated the greatest amount of administered dose with a lower activity in heart, muscle, brain and red blood cells. Radioactivity in liver and kidney rose slightly during weeks 2 through 4 after injection and decreased in spleen.

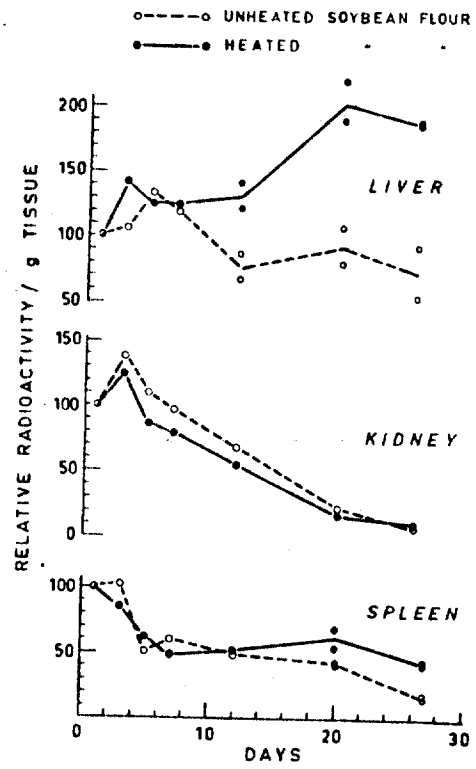

Fig. 2 Radioactivity of kidneys, liver and spleen of rats fed a diet of heated or unheated soybean flour and injected with labeled vitamin B_{12}. Values are expressed in terms of percentage of the dosage present in 1 g of tissue relative to that of 1 day after injection (100%). Each point is the mean of two rats, except for liver on days 12, 20, and 27 and for spleen on days 20 and 27, where each point indicates the value for one rat.

It appears, therefore, that the increased requirement for vitamin B_{12} in U rats can be attributed to two factors, a decreased availability of the vitamin formed by the intestinal flora and possibly an increased turnover of the absorbed vitamin.

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Original Communications

Rat Liver Glutamate Formiminotransferase Activity in B_{12} Deprivation and Germfree Conditions[1, 2, 3]

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INCREASED URINARY EXCRETION of formiminoglutamic acid (FIGLU) by rats fed vitamin B_{12}-deficient diets has been demonstrated (1–3). The conversion of FIGLU to glutamic acid requires tetrahydrofolate (THF), which serves as the acceptor for the formimino group transferred by the enzyme glutamate formiminotransferase. The N^5-methyl tetrahydrofolate (N^5-methyl-THF) is the primary storage form of folic acid in the body. A series of investigations (4–6) have defined the pathway by which N^5-methyl THF is converted to THF. This pathway is blocked in B_{12} deficiency so that THF may not be available to accept the formimino group and FIGLU is excreted. Baker et al. (7) suggested, and Vitale and Hegsted (1) demonstrated, that during B_{12} deficiency liver glutamate formiminotransferase activity is decreased. In contrast, Ohara et al. (8) and Kutzback et al. (9) reported no significant decreases in the liver level of glutamate formiminotransferase in rats fed vitamin B_{12}-deficient diets. Vitale and Hegsted (1) offered three proposals as possible explanations for their observation that liver glutamate formiminotransferase activity is decreased during B_{12} deficiency: *1*) vitamin B_{12} may be required for the synthesis or activation of the enzyme; *2*) the synthesis of the enzyme may be depressed when the level of the substrate, THF, is low; or *3*) competition between N^5-methyl THF and THF for the enzyme may influence either the activity or synthesis of the enzyme. The present study was designed to determine if one of the proposals explains the decrease in liver glutamate formiminotransferase activity during B_{12} deficiency.

METHODS

Three experiments were designed to study the effects of vitamin B_{12} and folate metabolites on the activity of liver glutamate formiminotransferase. The diets contained 15% extracted peanut meal and were low in methionine, choline, and B_{12} (Table 1). Lysine and threonine, which are limiting in peanut meal, were added to the diet. The diets used in *experiments 2* and *3* differed from those of *experiment 1* in that glucose was replaced by cornstarch and thiamin levels were increased five-fold before sterilization by autoclaving. In *experiment 1* there were four groups of six male rats weighing 42 g; *group 1* was given the diet supplemented with 20 μg B_{12}/100 g diet;

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[4] Department of Foods and Nutrition, University of Missouri, Columbus, Missouri. [5] Tufts University School of Medicine, Boston, Massachusetts.

TABLE I

Effect of B_{12} on weight gain, serum and liver B_{12} levels, total folates and THF, liver glutamate formiminotransferase activity, and FIGLU excretion

Experiment	Group	n	B_{12} Supplement	Weight gain, g	Vitamin B_{12}		Serum folates		Glutamate formiminotransferase activity, mμmoles gram protein × 10^{-3}	Urinary FIGLU, μg/day	
					Serum, pg/ml	Liver, pg/g	*L. casei* folates, ng/ml	*S. faecalis* folates, ng/ml			
1[a]	1	6	20 μg/100 g	144 ± 7[e]	1,126 ± 60	7,587 ± 340	107 ± 20	39 ± 3	7.70 ± 0.70	243 ± 15	
	2	6	0	92 ± 5[P<0.025][f]	15 ± 3[P<0.005]	1,363 ± 56[P<0.001]	119 ± 8[NS]	29 ± 3[NS]	7.05 ± 0.70[NS]	311 ± 36[P<0.025]	
	3	6	B_{12} injection	159 ± 1	1,059 ± 40	7,252 ± 222	66 ± 3	41 ± 3	8.65 ± 1.05	160 ± 33	
	4	6	Saline injection	89 ± 3[P<0.001]	51 ± 10[P<0.005]	1,431 ± 170[P<0.001]	132 ± 8[P<0.005]	33 ± 5[NS]	5.20 ± 0.70[P<0.025]	335 ± 51[P<0.01]	
2[b]	1[d]	6	2 μg/100 g	103 ± 3	322 ± 8	15,252 ± 1,958	66 ± 6	30 ± 3	4.75 ± 0.45		
	2[d]	6	0	90 ± 4[P<0.05]	108 ± 21[P<0.005]	5,349 ± 168[P<0.025]	72 ± 9[NS]	48 ± 5[P<0.05]	8.30 ± 0.75[P<0.025]		
										Liver folate	
3[c]	1	9	2 μg/100 g	300 ± 6	336 ± 34	9,325 ± 758	144 ± 8	41 ± 4	5.85 ± 0.70	Total, ng/g: 10,350 ± 1,693	THF, ng/g: 830 ± 63
	2	9	0	194 ± 2[NS]	76 ± 10[P<0.005]	4,202 ± 535[P<0.001]	139 ± 15[NS]	46 ± 4[NS]	10.45 ± 0.95[P<0.005]	15,000 ± 3,341[NS]	456 ± 29[P<0.05]
	3[d]	8	2 μg/100 g	222 ± 6	180 ± 18	6,677 ± 828	77 ± 4	32 ± 5	6.50 ± 0.75	16,150 ± 927	383 ± 45
	4[d]	8	0	216 ± 5[NS]	58 ± 6[P<0.025]	8,597 ± 563[NS]	93 ± 6[P<0.005]	48 ± 3[P<0.025]	7.30 ± 1.10[NS]	16,200 ± 2,557[NS]	556 ± 73[NS]

NS = not significant. [a] Fifty-one days duration. [b] Twenty-one days duration. [c] Fifty-one days duration. [d] Germfree rats. [e] Standard error of mean. [f] Level of significance.

group 2, the B_{12}-deficient diet and weekly intraperitoneal injections of 20 μg B_{12}; *group 3*, the B_{12}-deficient diet; and *group 4*, the B_{12}-deficient diet and weekly intraperitoneal injections of saline. This experiment lasted 51 days. In the second experiment, two groups of six germfree male rats weighing 86 g were maintained in isolators for 21 days. One group was fed the B_{12}-deficient diet and the other, the diet supplemented with 2 μg B_{12}/100 g diet. In the third experiment, two groups of nine male rats weighing 45 g were maintained under conventional conditions for 51 days and two groups of eight germfree rats weighing 77 g were maintained in isolators for the same period. One group of each of the conventional and germfree rats was fed the diet deficient in B_{12} and one group of each, the diet supplemented with 2 μg B_{12}/100 g diet. The animals were killed by ether anesthesia and blood was obtained from the abdominal aorta. The livers were homogenized in sucrose solution and the soluble fraction was retained. The following determinations were done: hemoglobin (10), hematocrit by the microcapillary method, serum folate and liver folate (*experiment 3*) using *Streptococcus faecalis* and *Lactobacillus casei* assays (11), serum and liver vitamin B_{12} utilizing *Euglena gracilis* as the test organism (12), and liver glutamate formiminotransferase activity (13). Urine was collected from the rats in *experiment 1* for 3 days immediately prior to death and was analyzed for FIGLU (13). The feces from germfree rats were collected on the final day of the experiment and were plated on agar; no evidence of bacterial contamination was noted. The cecums in these rats were only slightly enlarged.

RESULTS

The treatments had no effect either on hemoglobin concentrations or hematocrits, but weight gains were increased by B_{12} supplementation (Table 1). Of interest is the weight gain effected by injection of B_{12} (*experiment 1, group 3*) as compared to that effected by dietary B_{12} (*experiment 1, group 1*). In all groups of rats, serum and liver levels of B_{12} were significantly higher when B_{12} was given, with the exception of the livers of the germfree rats of *experiment 3*. Serum total folates as determined by *L. casei* assay were lower when B_{12} was given except for the conventional rats of *experiment 3* in which both groups had similar serum total folate levels. Serum total folates were significantly higher ($P <$ 0.01) in the conventional rats than in germfree rats in both the B_{12}-restricted and dietary-supplemented groups. However, in *experiment 1* rats given intraperitoneal injections of B_{12} had significantly ($P < 0.0$[illegible] lower serum total folate levels than either the rats given B_{12} in the diet or the rats deprived of B_{12}. No differences were noted in the total folate concentration, as determined by *L. casei*, between the livers of the deficient rats and those given minimal amounts of B_{12}. The serum *S. faecalis* folate levels, presumably THF, were higher in rats given 20 μg B_{12} diets or injections than in B_{12}-deficient rats. In contrast, rats fed the diet containing 2 μg B_{12}/100 g had lower serum *S. faecalis* folate levels than did the deficient animals. Minimal supplementation with B_{12} (*experiment 3*) caused opposite effects on the liver *S. faecalis* folate levels of the conventional and germfree rats. The B_{12}-supplemented conventional rats had significantly ($P < 0.005$) higher concentrations of liver *S. faecalis* folate than the deficient ones while the B_{12}-deficient germfree rats had a higher liver *S. faecalis* folate concentration than the B_{12}-supplemented rats. In rats given the 20 μg B_{12}, the ratio of serum *S. faecalis* folates to *L. casei* folates was greater than that of the deficient rats, but in *experiments 2* and *3*, the ratio of serum *S. faecalis* folates to *L. casei* folates was greater in the nonsupplemented groups. The specific activities of the liver enzyme, glutamate formiminotransferase, were rather constant in these experiments. In the first experiment, the rats fed the diet supplemented with 20 μg B_{12} tended to have higher specific activities of the enzyme than the deficient rats and rats given B_{12} by injection had significantly higher formiminotransferase activities than did the

nonsupplemented rats ($P < 0.025$). In the other experiments higher levels of liver glutamate formiminotransferase activity were observed in rats when the diet without B_{12} was fed rather than in the rats given the 2-μg B_{12} diet both in germfree and conventional rats.

The data for the serum *S. faecalis* folates and *L. casei* folates were fitted to regression equations for average daily weight gain and liver glutamate formiminotransferase activity. The regression of growth rate on the ratio of serum *S. faecalis* folates to *L. casei* folates was significant at $P < 0.01$. The regression of liver glutamate formiminotransferase activity on the serum *S. faecalis* folates was significant at $P < 0.025$.

DISCUSSION

Conventional rats given adequate amounts of B_{12} had higher serum *S. faecalis* folates, lower serum *L. casei* folates, and increased ratios of *S. faecalis* folates to *L. casei* total folates than nonsupplemented rats. In contrast, rats fed diets supplemented with only 2 μg B_{12}/100 g had lower serum *S. faecalis* folates and lower ratios of *S. faecalis* folates to *L. casei* folates and, as before, lower total folates than the nonsupplemented rats. The effects of B_{12} dietary supplementation on folate metabolism were markedly different from that of B_{12} supplementation by injection, which more closely resembled the response of germfree rats to B_{12} supplementation. The effect of B_{12} on the intestinal microflora was not investigated.

The data indicate that it is unlikely that the activity of the liver enzyme, glutamate formiminotransferase, is depressed simply by B_{12} deficiency as suggested by Vitale and Hegsted (1). The data also indicate that *S. faecalis* folates and N^5-methyl THF are not in competition for binding sites on the enzyme.

Vitale and Hegsted (1) also suggested that the synthesis of glutamate formiminotransferase may be depressed when the level of *S. faecalis* activity is low. Although the data presented here do not disprove this possibility, the observations of Taylor et al. (6) suggest that other factors are involved. Using a methionine-B_{12} auxotroph of *E. coli*, they found that the common intermediate in the biosynthesis of methionine, thymidine, the purines, and serine is $N^{5,10}$-methylene THF dehydrogenase and is subject to repression by end products. An enzyme with a function similar to glutamate formiminotransferase, serine hydroxymethylase, is also subject to end-product repression but to a much lesser degree, the magnitude being about the same as shown in these experiments and those of Vitale and Hegsted (1) for formiminotransferase.

Growth was not severely depressed in the second and third experiments in this study, indicating that THF could be regenerated by processes other than the B_{12} dependent N^5-methyl THF—homocysteine methyl transferase system. When growth was depressed as in *experiment 1*, these pathways would be repressed and N^5-methyl THF would accumulate as shown by Herbert and Zalusky (14) and FIGLU would be excreted as shown here. This is supported by data presented here, which does indicate that the ratios of serum *S. faecalis* folates to *L. casei* total folates are closely related to growth rates.

In line with the conclusions of Ohara et al. (8) and Kutzback et al. (9) no evidence is presented to indicate that B_{12} directly influences the activity of the liver enzyme, glutamate formiminotransferase. It seems reasonable to suggest that the control mechanisms for glutamate formiminotransferase are similar to those described for serine hydroxymethylase (6).

SUMMARY

The activity of the liver enzyme, glutamate formiminotransferase, has been reexamined in B_{12} supplementation and in

germfree conditions. The data indicated that under conventional conditions, B_{12} supplementation resulted in increased serum *S. faecalis* folate, decreased serum *L. casei* total folate levels, and increased liver glutamate formiminotransferase activity. Using essentially the same diet supplemented with minimal amount of B_{12} and using germfree animals, results were obtained indicating that the controls of folate metabolism and liver glutamate formiminotransferase activity are not under direct influence of B_{12} supplementation.

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